Effects of feeding 3-nitrooxypropanol to lactating dairy cows on methane emissions, animal performance and rumen fermentation

by

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Abstract

Methane emissions from ruminants are of great concern as they contribute to greenhouse gasses emitted within the atmosphere. The objective of this research was to evaluate a novel biochemical compound, 3-nitrooxypropanol, on its ability to reduce methane emissions and its impact on animal performance and rumen fermentation when fed to lactating dairy cows. In Study 1, feeding 2,500 mg/d 3-nitrooxypropanol in a 38% forage diet reduced methane emissions by 60% and increased body weight gain. In Study 2, feeding 1,250 and 2,500 mg/d 3-nitrooxypropanol in a 60% forage diet reduced methane emissions by 23 and 37%, respectively, and increased nutrient digestibility. In both studies, feed intake and animal performance were not negatively affected. These findings suggest that 3-nitrooxypropanol can reduce methane emissions from lactating dairy cows without adverse effects.

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List of Abbreviations

ADF	Acid detergent fibre
BES	α-Bromoethanesulfonic acid
BHBA	Beta-hydroxybutyric acid
BW	Body weight
CH_4	Methane
CO_2	Carbon dioxide
CON	Control treatment study 1 and study 2
CP	Crude protein
DE	Digestible energy
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
dOM	Digested organic matter
ECM	Energy corrected milk
FCM	Fat corrected milk
GC	Gas chromatography
GE	Gross energy
GEI	Gross energy intake
GHG	Greenhouse gases
GWP	Global warming potential
HIGH	Study 2: 2,500 mg/d 3-nitrooxypropanol treatment
LCFA	Long-chain fatty acids
LOW	Study 2: 1,250 mg/d 3-nitrooxypropanol treatment
ME	Metabolizable energy
MetHb	Methemoglobin
MP	Metabolizable protein
MUFA	Monounsaturated fatty acids
NAD^+/NADH	Nicotinamide adenine dinucleotide
NDF	Neutral detergent fibre

NEFA	Non-esterfied fatty acids
NFC	Non-fibre carbohydrates
NH_3-N	Ammonia nitrogen
NOP	3-nitrooxypropanol
NSC	Non-structural carbohydrates
OM	Organic matter
PUFA	Polyunsaturated fatty acids
PUN	Plasma urea nitrogen
RFI	Residual feed intake
RFID	Radio frequency identification
SEM	Standard error of the mean
SF_6	Sulphur hexafluoride
SiO_2	Silicon dioxide
TMR	Total mixed ration
VFA	Volatile fatty acids

1.0 Literature Review

1.1 Introduction

The temperature of the air surrounding the earth is increasing, a process referred to as global warming. One of the main focuses to reduce such effects is to reduce the amount of greenhouse gases emitted into the atmosphere that act to trap heat. Greenhouse gases (GHG) are emitted from several different sources, both natural and artificial; however the two sources with the most emphasis are the agricultural and fossil fuel industries. Within agriculture, great emphasis is placed on methane (CH_4) emissions from ruminants, in particular cattle. As such, the purpose of this literature review is to outline greenhouse gas production, how methane is produced by ruminants and methods to reduce emissions.

1.2 Greenhouse Gas Production

1.2.1 Global Warming Overview

Global warming refers to an increase in the temperature near earth's surface due to an increasing amount of GHG present in the atmosphere, and is a component of climate change. Greenhouse gases: carbon dioxide, CH_4, nitrous oxide, and fluorinated gases, act to trap heat in the atmosphere (EPA, 2014; Knapp et al., 2014). The extent to which each gas contributes to global warming is dependent on several factors such as how much gas is present, the concentration of gas present, how long the gases stay in the environment, and how effective they are at absorbing energy. Each GHG is given a Global Warming Potential (GWP), a measure of how much energy it absorbs over a period time and is compared to carbon dioxide. A higher GWP indicates the gas causes

more warming. Over 100 years, the GWP of carbon dioxide is 1, methane 25, nitrous oxide 298 and varies for different fluorinated gases (IPCC, 2014).

1.2.2 Global Greenhouse Gas Emissions

Globally, CH_4 is the most abundant GHG other than carbon dioxide and the natural GHG water vapor, and is released from both anthropogenic and natural sources (Wuebbles and Hayhoe, 2002). Anthropogenic sources consist of emissions from agriculture, waste disposal, and extraction of fossil fuels whereas natural sources can arise from wetlands, wild ruminants and oceans. Livestock contributes 12% of total anthropogenic sources (Havlik et al., 2014). In addition, agriculture is responsible for 90% of anthropogenic nitrous oxide emissions (DeAngelo et al., 2006) with the main source being soil through the processes of nitrification and denitrification. Because of this, and the increasing awareness of the effect GHG have on the environment, there is an increased demand to reduce emissions from all facets of daily life, in particular the energy and agricultural sectors.

1.2.3 Canada's Emissions

According to the National Inventory Report published by Environment Canada (2014), in 2012 Canada's total GHG emissions were an estimated 699 megatonnes of carbon dioxide equivalent; 79% from carbon dioxide, 13% from CH_4, 7% from nitrous oxide and 1% from fluorinated gases. Of this, agriculture contributed 10% compared to 80% from energy sectors. However, agriculture contributes 22% of the national CH_4 and 74% of the national nitrous oxide emissions. Historically, a majority of emissions were due to livestock production; however, from 2005-2012 emissions have decreased from 67 to 57% of total agricultural emissions due to a decline in livestock numbers. Despite this,

there is a great demand to further reduce emissions from livestock, in particular ruminants as enteric CH_4 production accounts for 56% of all livestock emissions.

1.2.4 Animal Agriculture

In order to reduce the environmental impact of ruminants, emphasis has been placed on reduction of CH_4 and nitrous oxide rather than carbon dioxide. Carbon dioxide generated is done so through respiration and digestive processes in livestock however, this carbon dioxide can be reabsorbed by plants meaning that it is a continuous cycle. This means that no net carbon dioxide is produced (Pitesky et al., 2009).

Where animal agriculture is concerned, nitrous oxide production is mostly through nitrification and denitrification of organic nitrogen found in animal urine and manure. While manure can re-enter the production cycle if applied as fertilizer, nitrous oxide losses range from 0-5% of nitrogen applied as manure and indirect losses occur from runoff and leaching of nitrogen (Pitesky et al., 2009).

Methane is primarily produced by ruminant animals from enteric fermentation; however secondary sources can come from manure decomposition (Mosier et al., 1998; Pitesky et al., 2009). Mitigation of CH_4 produced due to fermentation can be mitigated through animal management techniques; however, it is important to consider the entire animal production cycle when making managerial change to ensure production of CH_4 is not shifted to a different area in the cycle.

1.3 Methanogenesis

1.3.1 Fermentation

While microbial digestion normally occurs at the end of the digestive tract, ruminants have a large anaerobic fermentation chamber located at the beginning of the digestive tract referred to as the rumen (Moss et al., 2000); allowing for greater efficiency in digesting carbohydrates and plant cell walls. Feed macromolecules are degraded by enzymes of endogenous and microbial origin into smaller molecules that can be absorbed into the blood and utilized by the animal. In the rumen, anaerobic fermentation of plant components occurs; where the end products are volatile fatty acids (VFA), carbon dioxide, CH_4 and microbial cells (Prins, 1979). Briefly, the pathways involved for fermentations and methanogensis, as described in the literature (Prins, 1979; Immig, 1996; Moss et al., 2000; Bell and Eckard, 2012), are as follows.

Plant components such as starch, cellulose, pectin and hemi-cellulose are broken down by microbial hydrolytic enzymes to glucose equivalents which enter glycolysis to form pyruvate:

$$\text{Glucose} \rightarrow 2 \text{ pyruvate} + 4H$$

The fermentation of pyruvate, under anaerobic conditions, results in production of reduced co-factors such as NADH which are re-oxidised to NAD^+ to complete synthesis of VFA. Different bacteria, protozoa and fungi have different strategies for reoxidizing NADH. The hydrogen from NADH can be used to form butyrate or pyruvate, while pyruvate and NADH can be used to synthesize propionate (Immig, 1996). The most common pathway that does not consume hydrogen is formation of acetate from pyruvate via acetyl-CoA, and while not common in the rumen, some species can form lactate or

ethanol from pyruvate. In addition, during the conversion of pyruvate to acetyl-CoA, formate and CO_2 can be produced and become substrates for methanogenesis (Prin, 1979).

Pyruvate + H_2O → acetate (C2) + CO_2 + 2H

2C2 + 4H → butyrate (C4) + $2H_2O$

Pyruvate + 4H → propionate (C3) + H_2O

Reoxidation results in the formation of the above end products; however, H_2-producing organisms have the ability to regenerate NAD^+ through H_2 gas formation. While production of hydrogen in the rumen is an unfavorable process, it occurs when the partial pressure of hydrogen is low. Molecular hydrogen acts as a feedback inhibitor (Immig, 1996) and must be removed from the rumen. Fortunately hydrogen does not accumulate in the rumen due to the relationship between fermenting species and hydrogen utilising bacteria (eg. methanogens); the relationship being referred to as "interspecies hydrogen transfer".

The main hydrogen utilising pathway in the rumen is through formation of CH_4 by methanogens:

$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$ (methanogenesis)

If H_2 is not removed from the rumen, NADH can be reoxidized by the fermenting bacteria present to ethanol or lactate indicating ruminal upset (Moss et al., 2000), and a reduction in organic matter digestion and microbial growth can occur (Mathison et al., 1998).

From the equations above, one can see that the process of glucose breakdown and acetate formation is a hydrogen producing reaction whereas formation of propionate,

butyrate, and CH_4 are hydrogen consuming. This means that the molar percentage of VFA influences production of CH_4 through production and consumption of hydrogen (Johnson and Johnson, 1995; Moss et al., 2000). Accounting for the stoichiometry of hydrogen in ruminal metabolism, the equations above can be organized such that:

$$4\ CH_4 = 2\ C2 - C3 + 2\ C4 \text{ (Moss et al., 2000)}$$

An increase in acetate and butyrate promotes CH_4 production, while propionate acts as a competitive pathway. It should be known that, while on its own, production of butyrate consumes hydrogen, overall the formation of butyrate produces both hydrogen and carbon dioxide as pyruvate must be degraded to acetate prior to butyrate being formed.

1.3.2 Methanogens

Methanogens belong to the domain *Archaea* and the phylum *Euryarchaeota* and are different from *Bacteria* as they lack a peptidoglycan in the cell wall (Hook et al., 2010; Attwood et al., 2011). Strain of methanogen present in the rumen can differ greatly depending on diet (Whitford et al., 2001; Zhou et al., 2010), sampling location (Shin et al., 2004), and animal environment however, it appears that *Methanobrevimacter* spp. contributes two-thirds of rumen Archaea (Morgavi et al., 2010). Methanogens are generally found in rumen fluid and on solid digesta but some have been isolated from the rumen epithelium.

Methanogens grow at a neutral pH, between 6-8, and utilize CH_4 formation as a mechanism of generating ATP (Mathison et al., 1998; Hook et al., 2010). While substrates such as acetate, methanol, mono-, di- and tri- methylamine are available for

CH_4 production, it appears as though only H_2/CO_2 and formate, to a lesser extent, are used in the rumen (Mathison et al., 1998; Moss et al., 2000; Hook et al., 2010). Common characteristics of methanogens are that they have coenzyme F_{420} a cofactor necessary for enzymes such as hydrogenase and formate dehydrogenase, and coenzyme M which is either produced by methanogens or required from an external source (Rouvière and Wolfe, 1988; Hook et al., 2010).

Methanogens have symbiotic relationships with many microorganisms in the rumen where interspecies hydrogen transfer is concerned. An example of this described by Prins et al. (1979) is *Ruminococcus albus*. Through CH_4 formation by methanogens, the partial pressure of H_2 is kept low such that *R. albus* can continue to produce H_2 gas and attain more energy through formation of acetate and ATP. Another important symbiotic relationship found is that between methanogens and protozoa. Methanogens can exist both in and on protozoal cells. Intracellular methanogens can account for 1-2% of a host protozoa's cell volume while methanogens on the cell surface are less abundant and vary with diet and feeding time (Hook et al., 2010; Morgavi et al., 2010). While the exact relationship between methanogens and protozoa is not understood, it is believed that protozoa serve as hosts for methanogens, protecting them from oxygen toxicity (Morgavi et al., 2010). Because of this relationship, it has been suggested that mitigation strategies could target protozoa as elimination of protozoa has been successful in reducing CH_4 emissions

1.3.3 Effects on Animal Production

Volatile fatty acids provide ruminants with over 70% of their energy with acetate, butyrate and propionate being utilized by epithelial cells or absorbed across the ruminal

epithelium and transferred through the body in the blood (Bergman, 1990). The relationship between CH_4 and VFA shows that acetate formation promotes CH_4 production while propionate is a competitive pathway. On average, 6% of ingested energy is lost to CH_4 (Johnson and Johnson, 1995) however this can vary from 2-12% depending on the diet fed.

While great emphasis has been placed on CH_4 mitigation for environmental reasons, production of CH_4 in the rumen takes away energy from the animal that could otherwise be used for production. Where energy flow is concerned, gross energy (GE) is the total energy of a feed. GE minus the energy lost to feces is referred to digestible energy, and GE minus energy lost to feces, urine and gas is referred to as metabolizable energy. In general, the energy lost to feces and urine is easy to measure however measuring the output to gasses is more complicated (Blaxter and Clapperton, 1965) and will be highlighted in the next section.

1.4 Methane Measurement from Ruminants

1.4.1 Chamber Technique

The use of chambers is often considered the "golden standard" for CH_4 measurement (Pinares-Patiño et al., 2011) whereby all other ways of CH_4 emission determination are compared. The basic principle of chambers is to collect and measure all of the air exchanged by an animal whether it be exhaled, eructed or flatulated. There are two types of chambers used: closed circuit and open circuit, with open circuit being the most common (Storm et al., 2012).

Briefly, closed circuit chambers do not allow for any inflow of air into the chamber but rather measure the change in air composition (Turner and Thornton, 1966). Once the chamber is closed, air is pumped to a sampling circuit where gas analysis can be completed before returning back to the chamber. This allows for measurement to occur rapidly and immediately however, measurements cannot be completed over a long period of time as renewal of air is required (Turner and Thornton, 1966). Initial measurments of CH_4 emissions used closed circuit chambers (Blaxter and Clapperton, 1965) however they have historically been used for measurements in fowl and animals under 70 kg (Waring and Brown, 1965; Farrel 1972).

Open circuit chambers are most commonly used for ruminant animals with many different types and designs of chambers, all with varying levels of intricacy. The basic principle of open circuit chambers found in the literature (Miller and Koes, 1988; Johnson and Johnson, 1995; Klein and Wright, 2006) is that fresh air is taken from outside the chamber while air from inside the chamber is pumped out through a flow meter and different gas sensors. Additionally, the chamber is kept at a slight negative pressure such that any leaks are inward and a loss of air does not occur. In the case of CH_4, CH_4 emissions can be calculated by the difference present in air exiting the chamber versus entering.

The biggest advantage of using chambers for CH_4 measurement is that the animal is contained in a controlled environment whereby total digestive tract methane emissions can be accurately measured whether the source be eructation or flattulence (Pinares-Patiño et al., 2011). However, the effects of animal movement,diet selection and animal interaction in a natural environment cannot be accounted for thus making it difficult to

extrapolate data to free ranging systems with a large number of animals (Pinares-Patiño et al., 2011).

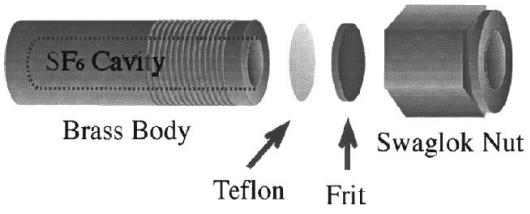
1.4.2 The SF_6 Tracer Gas Technique

In order to assess emissions while groups of animals remained in their natural environment, there was a need to develop a tracer technique for measuring CH_4. The tracer needs to have a constant and predictable release rate, have no impact on ruminal fermentation, be detectable at low concentrations, and be inert and nontoxic. Sulfur hexafluoride met the qualifications after rigorous testing and was chosen as a tracer gas for this technique to measure CH_4 emissions, in particular animals in grazing situations (Johnson et al., 1994; Johnson et al., 2007).

The SF_6 tracer gas technique uses a small permeation tube containing pure SF_6, with a known release rate of SF_6 that is placed into the rumen, and a halter fitted with capillary tubing (herein referred to halter) placed on the animal and connected to an evacuated sampling canister (herein referred to as a yoke; Johnson et al., 2007). The technique measures the ratio of SF_6:CH_4 in the air expelled from the animal through the nostrils and mouth by forceful contractions (Johnson et al., 1994; Beauchemin et al., 2012).

While construction of the materials used for the SF_6 technique can vary, in particular where halter and yoke design are concerned, the general requirements, as described by several authors (Johnson et al., 1994; McGinn et al., 2006; Johnson et al., 2007; Beauchemin et al., 2012), are as follows:

Permeation tubes are constructed using a brass rod with a hole drilled into one end to create a cavity for SF_6, a teflon window and stainless steel frit to allow for a continual and consistent permeation rate, and a swaglok nut to assemble the permeation tube (Figure 1-1). To fill a tube with SF_6, tubes are immersed in a cryogen, such as liquid nitrogen, the cavity in the tube filled with SF_6 gas followed by reassembly of the tube. As permeation rates are determined through a change in weight, it is imperative to have an accurate weight prior to and post filling with SF_6. In addition, weighing the tube for at least 5-6 weeks prior to placement in an animal is required to determine the permeation rate of an individual tube. Permeation tubes containing SF_6 are placed in the reticulorumen of animals using a balling gun or through a rumen cannula.



(Adapted from Johnson et al., 2007)

Figure 1-1 Permeation tube construction

Air expelled from an animal is sampled continually by capillary tubing over the animals nostril attached to a halter and collected into a yoke (Figure 1-2). Sampling rate of expelled air into yokes is determined by the length and diameter of capillary tubing between the inlet above the animal's nostrils and connection to the yokes. Stainless steel tubing acts as a flow restrictor and as such, duration of sampling can be determined by using either short or long tubing; however, yoke vacuum should only be reduced to 50% during a collection time in order to keep the fill rate constant. In order to maintain proper

function of capillary tubing, a filter is placed on the nose end of the tube to prevent dust and debris from entering the tubing, as well as additional tubing onto the end of the existing capillary tubing to prevent water from entering.

Yokes are commonly constructed of a PVC canister to collect sampled air and must be designed to allow for capillary tubing from the halter to connect into it, as well as be durable to prevent damage by animals. While there are several ways by which yokes can be designed, common types are those worn around animal's necks or placed on backpacks on an animal's back. For gas collection, yokes are evacuated prior to placing on an animal and following collection, yokes are removed and pressurized with pure nitrogen gas and gas samples can be transferred to a GC for analysis.

(Adapted from Johnson et al. (2007)

Figure 1-2: Animal wearing a halter and yoke

Both SF_6 and CH_4 emissions are determined by the assumption that emission rate is similar between SF_6 and CH_4 (Johnson et al., 2007). Therefore, emission rate of CH_4 (QCH_4) is calculated using measured CH_4 ($[CH_4]_y$) and SF_6 concentrations in samples, the known release rate of SF_6 (QSF_6), and background levels of CH_4 (CH_{4b}):

$$QCH_4=QSF_6\times([CH_4]_y - [CH_4]_b)/[SF_6] \quad \text{(Johnson et al., 2007)}$$

1.4.2.1 Validation of SF6 Technique

A concern with the SF_6 technique is its ability to calculate CH_4 emissions accurately from only measuring air expelled through the nostrils or eructed. Using sheep, Murray et al. (1976) determined 87% of CH_4 is produced in the rumen and 13% in the lower digestive tract. However, of all CH_4 produced >98% is eructed via the mouth and 2% in flatulence (Murray et al., 1976; Ulyatt et al, 1999; Pinares-Patiño et al., 2008a). Accurate use of this technique relies on the assumption that less than 5% of CH_4 is expelled via flatulence (Ulyatt et al, 1999).

Initial validation of the SF_6 technique by Johnson et al. (1994) using beef heifers and steers found no significant difference between CH_4 emission data when determined through use of a chamber versus SF_6. In addition, McGinn et al. (2006) found that while the SF_6 technique underestimated emissions by 4% compared to use of a whole animal chamber, the difference was not significant. In contrast, Boadi et al. (2002) found that with beef heifers, CH_4 emissions measured by the SF_6 technique were higher than that of a hood chamber, but not significantly.

While there are often numerical differences between CH_4 measurements using chambers and the SF_6 tracer technique, there is statistical agreement when beef cattle have been used. However there are greater discrepancies for lactating dairy cattle and sheep. Muñoz et al. (2012) used early lactation dairy cows and compared CH_4 emission data between the SF_6 technique versus chamber and found that the SF_6 technique overestimated emissions by 11 %, with a significant effect of experimental period as the study progressed, possibly due to increased time that permeation tubes were in the rumen.

However, Grainger et al. (2007) found the SF_6 technique to overestimate emissions by 2.3%, a value deemed insignificant.

In several studies using sheep, Pinares-Patiño et al. (2011) found that the SF_6 technique overestimated emissions when compared to chambers. For example, Pinares-Patiño et al. (2008b) found great variability in CH_4 emission detection with one trial showing emissions 39% higher than those measured in chambers and a second trial showing no difference. However, it is important to note that in the first trial, release rates of SF_6 were not specifically known as permeation tubes had been in sheep for 250 days prior to trial start date which could lead to an inaccurate calculation. The overestimation of emissions shown by both Pinares-Patiño et al. (2008b) and Muñoz et al. (2012) can be explained by an unknown permeation rate due to the permeation tubes being in the animal for prolonged periods of time.

Overall, in a meta-analysis conducted by Grainger et al. (2007), CH_4 emissions from beef cattle, dairy cattle, and sheep measured using the SF_6 technique resulted in emissions about 8% lower than those measured using a chamber, however deemed suitable with proper experimental design.

1.4.2.2 Other Considerations

While design of sampling equipment can be variable, it is critical to maintain consistency within a study to obtain representative results. Vlaming et al. (2007) reported that there was a positive relationship between permeation rate and calculated CH_4 emissions. For example, Pinares-Patiño et al. (2008a) found that within a study where permeation release rates ranged from 2.624 to 5.698 mg/d the effect of permeation rate

was more important on apparent CH_4 emission than dry matter intake. Assuming the relationship between release rate and CH_4 is linear, this difference in permeation rate would account for 8.5% difference in calculated CH_4 emission (Vlaming et al., 2007). In contrast, when permeation rates ranged from 2.214-3.594 g/d there was no significant effect of permeation rate on apparent CH_4 emission. As such, it is important to consider these effects when assessing individual animal emissions and when considering between animal variation.

A criticism of the SF_6 technique is its ability to detect CH_4 from ruminally cannulated animals due to the fact that CH_4 is measured from the nostrils of the animal and CH_4 could escape from the cannula. To address this concern, Beauchemin et al. (2012) conducted a study using beef cattle fitted with ruminal cannulas of different sizes and measured the SF_6:CH_4 in air collected from nostrils and at the cannula. Leakage of SF_6 and CH_4 was found regardless of cannula size and there was an increase in among-animal variability. The final recommendation was to avoid using cannulated animals but one should consider study design and objectives before fully discounting the use of cannulated animals.

As discussed, when employing the SF_6 tracer gas technique, consistency within a study is vital to is success. Despite its challenges with increased variability, it is a useful technique for obtaining data from a large number of animals but lacks the sensitivity to determine small changes in CH_4 emissions from cattle.

1.4.3 Carbon Dioxide Technique

Another tracer technique that can be employed is the CO_2 technique. This technique used naturally emitted CO_2 to estimate CH_4 emission (Storm et al., 2012). CO_2 and CH_4 can be measured directly or CH_4 emission calculated using the same equation for SF_6 using measured CO_2. The technique can be used at a whole barn level (Bjerg et al., 2012) or individual animal. The method is able to use portable equipment such as Gasmat (Gasmat Technologies Oy, Helsinki, Finland) to measure CO_2 concentrations and can be implemented in automated milking systems to measure emissions from individual dairy cows (Lassen et al., 2012). The main disadvantage of this technique is that CO_2 production is influenced by the animals' need for energy, meaning that size, activity and production of the animal influences production of CO_2.

1.4.4 GreenFeed

Another system used to measure emissions from livestock is GreenFeed (C-Lock Inc., Rapid City, South Dakota, USA). GreenFeed is a feeding station that animals visit periodically to receive a small amount of food. While at the system, they are identified by a radio frequency identification (RFID) tag and a fan pulls air into the system over the animals head and past its nose and mouth. The air is measured for airflow rates, gas concentrations and other environmental parameters which are then used to calculate the amount of a particular gas (commonly CO_2 or CH_4 emitted from the animal). The software collects data second-by-second such that measurements of both lung and rumen emissions of a particular gas from each eructation are measured while the animal is in the station. The system is designed to encourage animals to visit periodically throughout the day so that data from each feeding can be combined and analysed to determine individual animal or herd trends. The amount of feed used to entice animals to enter the system is

minimal to alleviate any confounding effects that it may have on emission data (C-Lock Inc., 2014). While publications using the GreenFeed system are minimal, preliminary results indicate that it is an acceptable way of measuring CH_4 emissions while keeping animals in their natural environment (Huhtanen et al., 2013) provided there are several visits to the station per day (Hammond, et al., 2013).

1.4.5 Micrometeorological Techniques

Micrometeorological techniques allow for measurement of CH_4 emissions outside without handling of animals or altering an animal's environment. These techniques measure the fluxes of gases in the atmosphere and relate them to animal emissions (Harper et al., 2011). Wind velocity and CH_4 concentration is determined for all micrometeorological techniques but the number of measuring points and theories used to calculate emission rates differ (Storm, 2012). Micrometeorological techniques available, as described by Harper et al. (2011), are as follows:

1) Mass balance techniques: measure all input and output emission within a specific volume with two methods being integrated horizontal flux and modified mass difference approach.
2) Vertical flux techniques determine emissions based on the vertical flux of gas above the ground and require the surface to be large and homogenous. Three methods are: flux gradient technique, eddy covariance and relaxed eddy accumulation.
3) Inverse dispersion techniques calculate a theoretical relationship between the source of emission and downwind concentration. The two methods are gaussian dispersion analysis and Lagrangian dispersion analysis (further

developed by Flesch et al. (1995) to the "backward" Lagrangian stochastic model (bLS dispersion model)).

4) Boundary layer budgeting is a technique whereby the accumulation of gas is measured within the boundary layer. The convective boundary layer develops during the day between ~100 m and 2 km above the surface and the nocturnal boundary layer that is only several meters above the surface at night. While this technique is not commonly used, there is interest in using it for measuring emissions from extensive grazing systems.

Often when micrometeorological techniques are used such as for grazing animals or large scale feedlots, there is an inability to determine the dry matter intake of the animal to provide an accurate CH_4 emission recorded per unit of feed intake. However, Tompkins et al. (2011) compared CH_4 emissions measured using the bLS dispersion model and open-circuit respiration chambers and found no difference in measured CH_4 emissions between the two techniques. Using micrometeorological techniques requires considerable expertise (McGinn, 2013); however they provide the ability to determine emissions from groups of animals within their natural outdoor environments.

1.4.6 In vitro Techniques

In vitro techniques of measuring rumen fermentation of feed and feedstuff have been used extensively for many years. The basic principle of *in vitro* systems is to allow for fermentation of feedstuff while maintaining an environment similar to the rumen (Storm, 2012). As such, feeds are incubated at 39° C with rumen fluid, buffer and minerals. Total gas production, and concentration of individual gases is measured thus resulting in *in vitro* production of CH_4. In the closed system, it is also possible to measure

degradability of feedstuff and short chain fatty acid production. *In vitro* techniques can include use of syringes (Bhatta et al., 2006), the rumen simulation technique (Rusitec; Czerkawski and Brechenridge, 1977) or closed batch fermentations.

In vitro techniques are useful for providing preliminary information on fermentation and commonly used when first assessing the effects that dietary additives would have on CH_4 production, but can be used to compare feedstuffs as well. There is generally good agreement between *in vitro* and *in vivo* techniques (Storm, 2012) however it is important to remember that CH_4 mitigation strategies should always be confirmed with *in vivo* measurements.

1.5 Reducing Methane Production from Ruminants

Many CH_4 mitigation strategies have been studied and while they have the potential to affect all domestic ruminants (beef cattle, dairy cattle, goats, sheep, etc.) a majority of research has been focussed on animals in confinement such as dairy and beef cattle or sheep for ease of sampling. Regardless of the mitigation strategy, it is important to ensure that they are cost effective and meet needs of farmers and animals.

Data for GHG production from ruminant agriculture are often misleading as production of GHG is affected by the number of animals, their productivity, and manure handling systems (Knapp et al., 2014). As such, CH_4 emissions should be referred to CH_4/unit of output or CH_4/unit of energy intake. This aids in ensuring that mitigation strategies do not negatively impact the animal such that CH_4 production is reduced and so is output.

1.5.1 Dietary Factors Affecting Methane Emissions

Feedstuff are composed of carbohydrates, protein, fat, water, and vitamins and minerals. In ruminants where their nutritional needs have been met, derivation of volatile fatty acids primarily occurs from carbohydrates and act as the primary source of energy. Carbohydrates can further be broken into basic components:

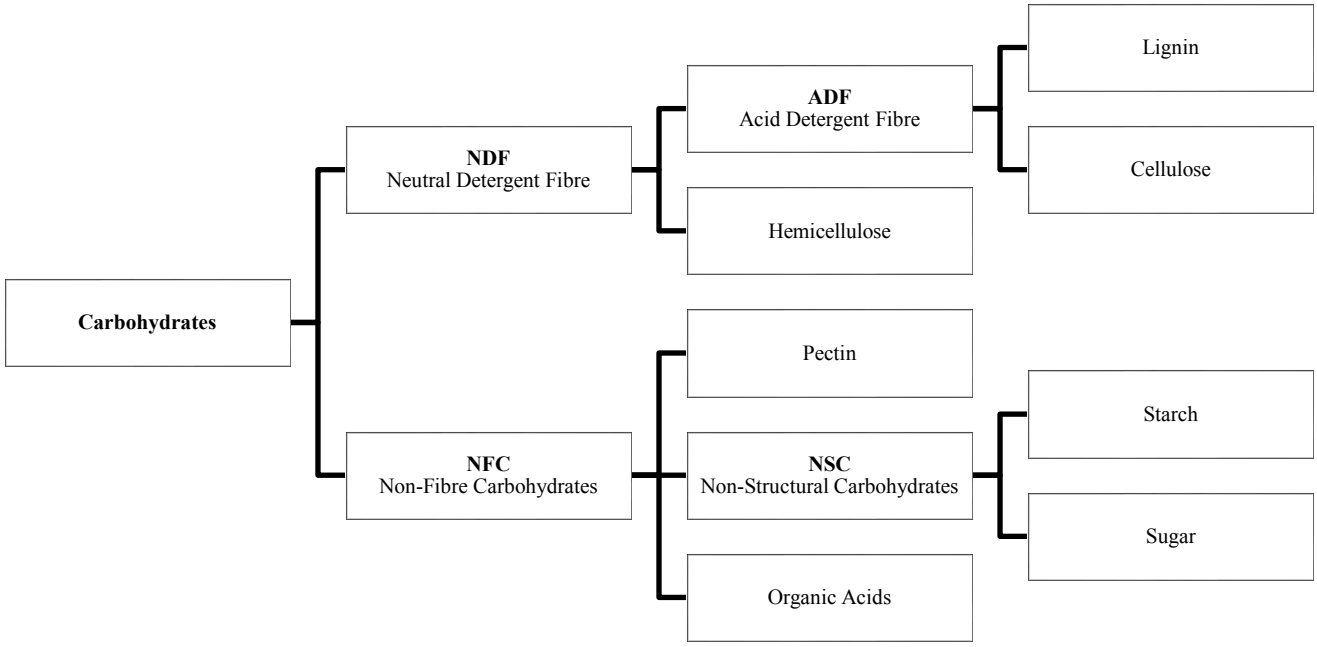


Figure 1-3: Schematic of carbohydrate structural components

Neutral detergent fibre refers to the structural components of plant cells with the exception of pectin, and non-fibre carbohydrates is the remaining carbohydrate fraction that is generally more readily fermented in the rumen. In general, forages are higher in NDF than NFC and grains are higher in NFC than NDF. This causes differences in feedstuff digestibility as plant chemical composition affects the amount of energy that can be extracted by microbes as well as the profile of VFA produced (Knapp et al., 2014).

1.5.1.1 Intake, Passage Rate and Digestibility

There is a relationship among feed intake, digestibility and passage rate where CH_4 production is concerned as the three are interlinked. Ingested feed is removed from the rumen by microbial degradation or passage to the lower gut (Bosch and Bruining, 1995) with passage being dependent on the chemical composition and physical property of the feed and rate of microbial degradation (Krizsan et al., 2010). If intake is increased, passage rate will increase which can cause a decrease in digestion that occurs in the rumen, regardless of the feeds initial ruminal digestibility. As dry matter intake increases, CH_4 production increases as there is more feed to be fermented but when expressed as a proportion of DMI or ingested GE, is reduced when intakes reach levels higher than maintenance requirements (Blaxter and Clapperton, 1965; Moe and Tyrell, 1979). On average, the percentage of GE lost to CH_4 decreases by 0.77 to 1.6% per level of intake (Johnson and Johnson, 1995; Beauchemin et al., 2006).

Processing of feeds, in particular forages, reduces particle size thus altering the rate of passage and at high intakes, a 20 to 40% reduction in CH_4 lost/unit of diet can be seen (Johnson and Johnson, 1995). Okine et al. (1989) found a 29% reduction in CH_4 production when weights were added to the rumen of steers resulting in an increased passage rate.

A meta-analysis conducted by Huhtanen et al. (2009) found that when intakes increase, dry matter digestibility tends to decrease but it is associated with a decrease in total-tract NDF digestibility, not with total-tract starch digestibility. This reduction in NDF digestibility results in a proportional reduction in CH_4 emission per unit of diet (Knapp et al., 2014) without a visible loss in animal productivity. Despite no reduction in

total-tract starch digestibility, there is a reduction in ruminal starch digestibility (Firkins et al., 2001). This is because starch can be digested in the small intestine or fermented in the hindgut. Hindgut fermentation will produce CH_4; however, will be proportionally less than what is eliminated from NDF fermentation in the rumen (Knapp et al., 2014). The effect to which this works is dependent on proportion of NDF and starch in the diet, however there is generally a decrease in net CH_4 production.

1.5.1.2 Type of Carbohydrate

It is well documented that dietary substrate affects the amount of CH_4 produced (Blaxter and Clapperton, 1965; Mathison et al., 1998; Beauchemin et al., 2008a) due to changes in ruminal pH and microbiota present (Johnson and Johnson, 1995); in particular through feeding of concentrates. While the definition of concentrate varies, it is commonly associated with the feeding of grain, a more digestible carbohydrate containing higher levels of starch when compared to forage. CH_4 production is reduced as starch ferments, rumen pH declines, and propionate production increases (Beauchemin et al., 2008a).

Diets that are high in hemicellulose, cellulose and lignin favor acetate and butyrate production (Moe and Tyrell, 1979) whereas starch based diets favor propionate production. However, diets with soluble sugars tend to stimulate butyrate production meaning they could be more methanogenic (Johnson and Johnson, 1995), rather than reducing methanogenesis. Ellis et al. (2012) modelled the effect that high-sugar grasses would have on CH_4 emissions and found that when expressed as a percentage of GE intake CH_4 increased however, when expressed as g/kg milk results were variable due to potential increases in milk production.

Feeding high grain diets results in CH_4 losses between 2-3% of GE (Johnson and Johnson, 1995). While considered positive where CH_4 emissions are concerned, this approach often comes with other challenges. High grain diets are often associated with acidosis or laminitis which can negatively impact animal production and welfare. In addition, feeding of high grain diets is not practical in all parts of the world, especially where grazing is the primary way of feeding animals.

1.5.2 Dietary Supplements

1.5.2.1 Lipid and Fatty Acid Supplementation

The main types of lipids that enter the rumen are triglycerides, phospholipids and galactolipids (Jenkins et al., 2009). Triglycerides consist of glycerol and three fatty acids. Fatty acids can be distinguished into two categories: unsaturated and saturated. Saturated fatty acids contain no double bonds while unsaturated fatty acids can be further distinguished into monounsaturated fatty acids (MUFA; containing one double bond) or polyunsaturated fatty acids (PUFA; more than one double bond). Phospholipids are a major component of cell walls and contain a diglyceride, a phosphate group and a simple organic molecule. Galactolipids are a part of plant membrane lipids and are a type of glycolipid whose sugar group is galactose.

Addition of lipids to cattle diets can act to reduce CH_4 emissions through different mechanisms including biohydrogenation of unsaturated fatty acids, enhanced propionic acid production, and protozoal inhibition (Johnson and Johnson, 1995). Biohydrogenation is the process of adding hydrogen atoms to a molecule to reduce or saturate organic compounds. In the rumen, biohydrogenation can act as an alternate hydrogen sink, but its

contribution as a hydrogen sink is minimal (Johnson and Johson, 1995; Jenkins et al., 2009) compared to CH_4. Unsaturated fatty acids have cytotoxic effects on some microbial species and the addition of hydrogen protects the microbial population (Jenkins et al., 2009). As such, inclusion of dietary fat is recommended to not exceed 6-7% of dietary DM as detrimental effects on dry matter intake and fibre digestion can occur (Coppock and Wilks, 1991; Allen, 2000; NRC, 2001). However, inclusion in diets has proved successful in reducing CH_4 emissions depending on the source, fatty acid profile, inclusion rate and diet composition (Beauchemin et al., 2008a). Some MUFA commonly added to diets are coconut oil, palm kernel oil, high-laurate canola oil or pure myristic acid whereas long-chain fatty acids (LCFA) sources are often oilseed and animal fats.

Machmüller et al., (2001) analysed the effect of coconut oil and lauric acic (MUFA equivalent to coconut oil) on CH_4 production using the RUSITEC technique with two basal diets differing in NDF content. Inclusion of coconut oil to the low NDF diet resulted in a reduction of fermentation of fibre and crude protein with no effect seen in the high NDF diet. In addition, CH_4 release was reduced by 62% when coconut oil was combined in the low NDF diet and not affected when combined with the high NDF diet. In contrast, lauric acid depressed CH_4 release by 78% regardless of basal diet. It appears that the primary mechanism by which MUFA reduce emissions is because of their toxicity to rumen methanogens (Machmüller et al., 2001), in particular with low NDF diets.

LCFA sources can be from pure oils or via crushed oilseeds however crushed oilseeds have less adverse effects on intake and fibre digestibility (Beauchemin et al., 2008b). Beauchemin et al. (2008b) compared tallow, sunflower oil and sunflower seeds

provided to beef heifers such that dietary fat content was 59 g/kg of DM with the basal diet consisting of primarily barley silage with steam rolled barley. DMI was reduced when sunflower seeds were fed with no difference seen between control, tallow or sunflower oil treatments, which led to sunflower seeds resulting in the greatest decrease in CH_4 loss (25% of GEI) when compared to tallow or sunflower oil (15% reduction expressed as % of GEI), however, when DE intake was considered, all lipid sources were effective at suppressing CH_4.

Beauchemin et al. (2008b) compiled a dataset of 17 studies using beef cattle, dairy cows and sheep and found that CH_4 (g/kg DMI) was reduced by 5.6% with each 1% addition of supplemental fat; however, there was variation in CH_4 reductions observed among fat sources. While Grainger and Beauchemin (2011) concluded that fat can be fed without reductions in animal production, potential implications on animal production are important considerations before implementation. In addition, cost effectiveness must be assessed.

1.5.2.2 Feed Additives

1.5.2.2.1 Tannins and Saponins

Methane emissions from cattle fed high-roughage diets are generally higher than those fed concentrates. As such, there is interest in looking at plant secondary metabolites, tannins and saponins for their effect on animal performance and CH_4 mitigation (Beauchemin et al., 2008a; Goel and Makkar 2012). Tannins are believed to reduce methanogenesis via two modes of action: directly by affecting populations of methanogens or indirectly by reducing hydrogen production (Tavendale et al., 2005;

Jayanegara et al., 2011; Goel and Makkar, 2012) while saponins are believed to work through their anti-protozoal effects (Beauchemin et al., 2008a).

The effect of tannins on CH_4 emissions is not consistent in the literature. Wischer et al. (2013) determined the effects of several tannin rich extracts (chestnut, grape seed, myrabolan, sumach and valonea) on CH_4 formation *in vitro* and found all extracts, with the exception of myrabolan to reduce CH_4 production (ml/g dOM). Addition of tannins reduced degradation of DM and OM in all cases. In comparison, Beauchemin et al. (2007) added condensed tannin from quebracho trees to the diet of beef heifers and steers and found no change in CH_4 emissions, DM, energy or fibre digestibility but digestibility of CP was reduced. Other studies have found inconsistent results where CH_4 reduction and digestibility are concerned (see: Goel and Makkar 2012 for a comprehensive review) and as such, further research is required into the efficacy and potential negative impacts of feeding tannins, such as reduced digestibility.

Tea saponins have been shown to have lasting antiprotozoal effects and inhibitory effects on methanogen activity (Wang et al., 2012) which would support their ability to reduce CH_4emissions, however like tannins, feeding saponins can decrease digestibility. A study by Holtshausen et al. (2009) added saponin containing Yucca schidifera (Yucca) or Quillaja saponaria (soap bark tree) to the diets of dairy cattle. While a previous *in vitro* study showed that both sources reduced CH_4 concentration, 24-h NDF digestibility was reduced. In the *in vivo* study, feeding saponin at 10 g/kg DM did not affect milk production, total tract digestibility, rumen fermentation or CH_4production, but DM intake was greater for cows fed saponin. In comparison, when saponins have been provided to sheep, there have been discrepancies between studies regarding the extent to which CH_4

is reduced and the effect on digestibility (eg. Hess et al., 2004; Santoso et al., 2004; Wang et al., 2009)

While effect of saponin and tannins on CH_4 emissions is inconsistent, further work to find a source effective at reducing emissions without anti-nutritional properties is desirable as a natural feed additive to reduce CH_4 emissions.

1.5.2.2.2 Ionophores

Ionophores are lipophilic substances that can accumulate in cell membranes to catalyze rapid ion movement and act as antiporters binding metal ions and creating a futile ion cycle resulting in a de-energized cell (Mathison et al., 1998; Russell and Houlihan, 2003). In general, gram-positive ruminal bacteria are more sensitive to ionophores compared to gram-negative species (Russell and Houlihan, 2003). The most commonly studied ionophore in cattle is monensin however others such as lasalocid, tetranasin, lysocellin, narasin, salinomycin and laidlomycin are used experimentally or commercially (Mathison et al., 1998). Monensin can be provided as a slow release capsule inserted in the rumen, or included in the diet as a premix (Beauchemin et al., 2008a).

Monensin is normally included in the diets of cattle to improve feed efficiency and prevent coccidiosis. However, monensin can decrease the acetate:propionate ratio and decrease ruminal protozoa numbers, which could contribute to its anti-methanogenic effect (Guan., 2006; Beauchemin et al., 2008a). Unfortunately, long term effects of monensin on CH_4 production are inconsistent and may not persist over long periods of time (Johnson and Johnson, 1995).

Guan et al. (2006) introduced monensin (33 mg/kg) to the diets of steers fed either a high- or low- concentrate diet and found that enteric CH_4 production (L/kg DMI or % GEI) was reduced by 27% for the high-concentrate diet in the first two weeks and by 30% for the low-concentrate diet in the first 4 weeks. However, emissions were restored to original levels by the third and sixth week for low- and high-concentrate diets, respectively. Grainger et al. (2010) found no difference in CH_4 production when monensin (450mg/cow/day) was added to the diets of lactating cows grazing ryegrass pasture and fed barley grain. However, Odongo et al. (2007) provided monensin to lactating dairy cattle fed a 60% forage diet and found CH_4 emissions (g/kg of BW) decreased by 9% and was sustained for 6 months. Therefore, inconsistencies in the efficacy of ionophores as a CH_4 mitigation strategy suggest that it is not a good long term approach for CH_4 emission mitigation.

1.5.2.2.3 Nitrate and Sulphate

Nitrate is a potential hydrogen sink in the rumen, however has not been thoroughly investigated due to its toxic effects on animals. In the rumen, nitrate is reduced to nitrite which is subsequently reduced to ammonia yielding more energy than methanogenesis (Mathison et al., 1998; van Zijderveld et al., 2010) and allowing for nitrate reducing bacteria to outcompete methanogens (Mathison et al., 1998). Absorbed nitrites can act to oxidize haemoglobin (Hb) to methaemoglobin (MetHb) and interfere with oxygen transport. The level at which nitrate causes toxicity is dependent upon the rate of nitrate consumption, type of feed, amount of carbohydrate fed and adaptation to nitrate (Vermunt and Visser, 1987), however diets should contain less than 0.45% nitrate N as near toxic levels of MetHb occur (Takahashi et al., 1998). van Zijderveld et al.

(2010) included nitrate at 2.6% dietary DM to sheep and found detectable amounts of MetHb in the blood of sheep however CH_4 was reduced by 32% when expressed as L/d and was still reduced when expressed as L/kg of DMI. In another study, van Zijderveld et al. (2011) fed 21 g nitrate/kg DMI to lactating Holstein-Freisan cows and found CH_4 was reduced by 16%. While there were no effects on milk yield or apparent digestibility of crude fat, NDF and starch, however, elevated MetHb levels were detected. While feeding nitrate or nitrites seems promising for reducing CH_4 emissions, the potential negative effects of toxicity do not make it a practical option.

Sulphur also acts as a terminal electron acceptor and sulphur reducers can use hydrogen at lower partial pressures thus making them able to outcompete methanogens when hydrogen levels are low (Mathison et al., 1998). However, sulfur tolerance in the diet is relatively low (NRC, 2001), suggesting little potential as a method to reduce CH_4 emissions.

1.5.3 Defaunation

Defaunation is the process whereby protozoa are eliminated from the rumen. As mentioned, protozoa have a symbiotic relationship with methanogens and some believe elimination of protozoa is a potential CH_4 mitigation strategy. Defaunation is not possible to be practically implemented in production settings however, for research, has been shown to reduce CH_4 emissions, in most cases. Some techniques used in research, as described by Jouany et al. (1988) are as follows:

1) Separating young animals from dams at birth: Ciliate protozoa have been found to be absent at birth and populate the rumen by 3 weeks after birth, or longer if

bacteria are not already established (Fonty et al., 1988). As such, if newborns are raised in isolation, little to no protozoa establish.

2) Introducing chemicals to the rumen: Introducing chemicals, such as copper sulphate, has been successful when combined with a starvation period, however, some chemical agents often result in mortality among animals
3) Emptying the rumen: Rumen contents are removed and the rumen is washed with water and formaldehyde to eliminate protozoa. However, in some cases if the formaldehyde is not rapidly washed from the rumen, animals can die.

Other techniques, as described by Hegarty (1999), which have potential to be investigated for implementation in production settings are through dietary manipulation, natural compounds and biological agents. Rumen protozoa are killed at low rumen pH meaning that high concentrate diets have the potential to reduce protozoa; however, a low rumen pH can have other detrimental effects on the animal. Some lipid sources have shown to reduce protozoal populations (eg. Machmüller et al., 1998), however as mentioned, their use can reduce digestibility. An example of natural compounds that have anti-protozoal effects is tea saponins (eg. Wang et al., 2012).

Biological agents that appear to have effects on protozoal populations in the rumen are probiotic additives such as *Saccharomyces cereviciae* and *Aspergillus oryae* (Hegarty, 1998). Active dry yeast and yeast cultures based on *S. cereviciae* are currently used within ruminant production systems to improve production however their effects on CH_4 emissions have not been extensively studied (Grainger and Beauchemin, 2011).

1.5.4 Improved animal production

A potential way of mitigating enteric CH_4emissions is genetic selection of animals with reduced CH_4/unit of diet as there is a great variation in enteric CH_4 emissions among animals, breeds and across time (Haas et al., 2011). Direct selection of animals with lower emissions is possible, however as measurement of emissions from all animals is difficult, improving traits associated with CH_4 emissions, such as residual feed intake (RFI) and improved productivity and efficiency, is promising (Wall et al., 2010; Haas et al., 2011; Knapp et al., 2014). As direct selection for reduced emissions has been utilized less due to difficulty in measurement, improved productivity and efficiency as well as RFI will be discussed briefly. However, with advances in measurement techniques, there is potential to select directly for reduced enteric emissions (Wall et al., 2010).

1.5.4.1 Productivity and Efficiency

Wall et al. (2010) cited two ways in which selection for productivity and efficiency can reduce emissions: 1) higher productivity tends to lead to higher gross efficiency through diluting the maintenance requirement of animals and 2) a certain level of production can be met with fewer higher yielding animals. As an example, Wall et al. (2010) compared two lines of dairy cattle. One selected for productivity with 17% higher milk yield per lactation and being 14% more efficient compared to a line of cows with average production levels in the United Kingdom. While the two groups of cows are fed two different diets due to their nutritional needs (high forage vs. low forage), an estimation of CH_4 yields from the two different groups of cows showed that the selected line produced 21% less CH_4 per kg of milk compared to non-selected cows in their first lactation.

Improved efficiency is seen in Canada across our dairy industry. Since 1990, emissions associated with the dairy industry have decreased 22% while there has been a 33% increase in average milk productivity (Environment Canada, 2014). Given the quota system in Canada, selecting animals for improved productivity has reduced the number of animals needed while meeting milk quota thus reducing emissions from fewer animals. A more elaborate comparison by Capper et al. (2009) paralleled dairy production in the United States in 2007 to production in 1944. The greatest difference was seen in the herd size: 25.6 million cows producing 53.0 billion kg of milk annually in 1944 compared to 9.2 million cows producing 84.2 billion kg. The reduction in animals and increase in production can be attributed to several factors, including, but not limited to: improved genetic selection for productivity and efficiency, improvement in nutritional management and overall herd management. When the whole production cycle is assessed, the carbon footprint per kg of milk in 2007 is 37% of that in 1944.

1.5.4.2 Residual Feed Intake

As cattle vary in nutrient requirements for the same level of production, there is interest in defining the trait of residual feed intake (RFI): the difference between actual feed intake and expected feed requirements for maintenance of BW and production (Koch, et al., 1963; Hegarty et al., 2007). Low RFI (– RFI) indicates animals are more efficient having lower levels of energy intake while maintaining levels of production compared to high RFI (+ RFI). The first evidence of – RFI having reduced CH_4 emissions showed that CH_4production was 28% less in – RFI compared to + RFI (Nkrumah et al., 2006). While the underlying theory is not fully known, they could have lower CH_4 emission due to reduced intake or difference in digestion patterns. Reduced

intake could mean increased retention time in the rumen resulting in greater digestion of carbohydrates and a greater supply of hydrogen for methanogens (Basarab et al., 2013). However, differences in feeding behavior, intake and ruminal retention time can influence microbial communities thus potentially causing a reduction in CH_4. For example, Zhou et al. (2010) found that there was no change in total methanogens present at – or + RFI, but the proportion of different species present was different. The difference in proportion of species present could influence CH_4 emissions by the substrates used in methanogenesis (Basarab et al., 2013); however CH_4 was not measured in the study.

1.5.4.3 Considerations for Production Enhancing Strategies

Advances in production have aided in reducing the environmental impact of animal agriculture (eg. Capper et al., 2007) however, to date, many of these advancements have been focussed on direct intervention, such as nutritional strategies including production enhancing agents. The use of production-enhancing agents can also act to indirectly reduce GHG emissions from livestock as they generally act in a way whereby less input is required to get an output, such as ionophores. While their impact on CH_4 emissions is variable and not consistent, they are used to improve feed efficiency which is economically justifiable (Mathison et al., 1998). Despite this, there is consumer concern regarding the effect of these compounds on the animal, as well as residues left in meat or milk, such that the European Union does not allow for them to be used (Beachemin et al., 2008a). The use of beta-agonists, a class of growth promotants, is being questioned and its use is banned in several countries. More recently, Health Canada announced it will phase out the use of antibiotics for growth promotion in livestock.

While the additives mentioned above do not have direct impacts on CH_4 mitigation, they aid in reducing emissions through improved production.

While consumers demand a more "natural" product free of additives, public opinion of the impact agriculture has on the environment is continually increasing and putting great pressure on agriculture to reduce emissions. In moving forward, we need to find strategies to reduce emissions from ruminants while being mindful of consumer demands. And, regardless of the mechanism by which animals are selected to be more productive and efficient, it is important to consider potential side effects including reduced reproductive capacity or increased health concerns and to minimize culling at all stages.

1.5.5 Novel biochemical compounds

Several strategies have been proven successful in mitigation of CH_4 emissions from ruminants; however few come without potential detrimental effects on production. As such, there is continual research into strategies that are practical and effective. Research into the use of biochemical compounds to reduce CH_4 emissions is continually ongoing. One compound that has been shown to be effective at inhibiting methanogenesis is α-Bromoethanesulfonic acid (BES; Gunsalus et al., 1978).

BES is a structural analogue to coenzyme M (2-mercaptoethanesulfonic acid) and is known to inhibit the methyl transfer reaction at the terminal step during CH_4 formation (Bouwer and McCarty, 1983). Because coenzyme M is only found in methanogenic bacteria (Mathison et al., 1998), BES can directly inhibit methanogenesis. Given the

ability of BES to inhibit methanogenesis, work has gone into synthesising similar compounds that have the same effects on ruminants.

A screening study conducted by Soliva et al. (2011) tested the effect of three synthetic compounds (levulinic acid, 3-azido-propionic acid ethyl ester and 4-[(pyridine-2-ylmethyl)-amino]-benzoic acid), *in vitro*. 3-azido-propionic acid was synthesized as a structural analogue to BES and drastically decreased CH_4 formation while the other two did not. Further to that, molecules substituted with nitrooxy groups have been identified as potential inhibitors of methanogenesis (Duval and Kindermann, 2012), such as 3-nitrooxypropanol (NOP), which *in vitro*, reduced methanogenesis by 86-95% (Martinez-Fernandez et al., 2014).

Furthermore, upon addition to the rumen of sheep (Martinez-Fernandez et al., 2014) and lactating dairy cows (Reynolds et al., 2014), CH_4 emissions were reduced without any negative effects on animal performance. Given the success of addition to the rumen, further studies were required to determine the effects if provided in the diet of lactating dairy cows, while housed in their perceived natural environment.

1.6 Summary

Although great efforts have gone towards finding strategies to reduce CH_4emissions from ruminants, there is still a need to find methods that do not impact animal production and are applicable at a global scale. The purpose of this thesis work is to further investigate a compound with the potential to mitigate CH_4emissions. The objectives of the two studies described in Chapters 2 and 3 were to determine the effects of 3-nitrooxypropanol on lactating Holstein cows when included into their daily TMR, as

would be done in an on-farm feeding scenario. CH_4 emissions, animal production, rumen fermentation and digestibility were assessed under high and low forage diets.

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2.0 STUDY 1: The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation[1]

2.1 Introduction

There is demand to reduce greenhouse gas emissions generated by the agricultural sector, in particular, the livestock industry. In recent years, it has been estimated that cattle alone are responsible for 11-17% of the methane generated globally (Beauchemin et al., 2009a). As methane has a global warming potential 21times that of carbon dioxide (United Nations, 2013), the environmental importance of emissions is self-evident. Another consideration is that between 2 and 12% of the ingested gross energy of cattle can be lost to methane (Johnson and Johnson, 1995); a loss of energy that could potentially be used by the animal. Enteric methane emissions from cattle can be reduced through dietary techniques such as improving forage quality, higher inclusion of concentrates in the diet and feeding lipids (Martin et al., 2010; Eckard et al., 2012). Additionally, previous research has shown that natural compounds such as tannins and saponins and synthetic dietary compounds such as ionophores can reduce methane emissions from ruminants through inhibition of methanogenesis or by shifting fermentation pathways to promote alternative hydrogen sinks, such as propionate production, thus reducing methane emissions (McAllister and Newbold, 2008; Martin et al., 2010).

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Haisan, J., Y. Sun, L. L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, D. R. Barreda, and M. Oba. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110-3119.

Recently, several molecules, substituted at various positions with at least one nitrooxy group, were identified as potential inhibitors of enteric methanogenesis (Duval and Kindermann, 2012). One such compound, 3-nitrooxypropanol (**NOP**) was developed from predecessor compounds (Ethyl 3-nitrooxy propionate; WO2011/070133 and 3-azido-propionic acid ethyl ester) which were identified from an in vitro rumen simulation screening assay (Soliva et al., 2011). The NOP exhibited a significantly higher potential to reduce methanogenesis in vitro than the well-known model compound bromoethanesulfonate (Soliva et al., 2011). Bromoethanesulfonate is a coenzyme M analog (Gunsalus et al., 1978) with very specific activity against methanogens that inhibits the reduction of methyl-coenzyme M to methane during the last step of methanogenesis (Immig, 1996).

Research using a rumen simulation technique showed that NOP is capable of reducing methane production (Romero-Perez et al., 2013a). Furthermore, when NOP was directly dosed into the rumen of sheep (Martinez-Fernandez et al., 2013) and lactating dairy cows (Reynolds et al., 2013) or fed once daily to beef cattle (Romero Perez et al., 2013b), methane emissions were reduced, and an increase in propionate concentration was observed suggesting a shift in rumen fermentation. However, the effect of NOP on methanogen numbers or microbial profile was not reported or consistently demonstrated in those previous studies.

Although use of NOP is a promising approach to reduce enteric methane emissions from ruminants, further studies are required to confirm its efficacy in reducing methane emissions while evaluating its effects on rumen fermentation and animal productivity. It was hypothesized that lactating dairy cows fed NOP would have reduced

methane emissions, and hence more energy would be available for milk production. The objectives of this study were to determine the effects of NOP on methane emissions, animal performance, rumen fermentation and rumen microbial profile of lactating dairy cows.

2.2 Materials and Methods

All procedures were pre-approved by the Animal Care and Use Committee for Livestock at the University of Alberta and conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada). The use of the biochemical compound (10% NOP on silicon dioxide (SiO_2)), developed by DSM Nutritional Products Ltd. (Kaiseraugst, Switzerland), in the diet of lactating dairy cows at 25 g/d was pre-approved by the Veterinary Drugs Directorate Division (Health Canada, Ottawa, ON) for research. According to their instructions, milk was discarded for the duration of the study and an additional 14-d milk withdrawal period was implemented upon completion of the study.

2.2.1 Experimental Design, Diet and Treatment

Twelve lactating Holstein cows with ruminal cannulas (Bar Diamond Inc., Parma, ID) were used in a crossover design study with 28-d periods consisting of 21 d of adaptation and 7 d of data and sample collection. Cows were separated into two groups based on the pre-experiment DIM (means ± SD: Group 1 = 100 ± 5.4, Group 2 = 76 ± 10.1). Group 1 had 4 multiparous and 4 primiparous cows and Group 2 had 2 multiparous and 2 primiparous cows, and they were randomly assigned to the treatment sequence. Pre-experiment BW (means ± SD) was 591.5 ± 58.9 and 567.5 ± 93.5 kg for Group 1 and 2, respectively. The study was conducted using two groups of cows to facilitate methane

measurement; we did not have sufficient supplies to measure methane emission for all cows at once, thus the whole study protocol was staggered by 7 d between the two groups. Cows were housed individually in tie stalls and milked twice daily in their stalls at 0400 and 1600 h.

All cows were fed the same diet as a TMR (Table 2-1), ad libitum, once daily at 0900 h, allowing for 5% refusals throughout the study, and had free access to water. The diet was formulated to provide adequate ME and MP for a 650-kg cow producing 40 kg milk per day (NRC, 2001) and assure ME and MP intake did not limit milk production of all cows. Cows were fed either SiO_2 as a control (**CON**) or NOP at 25 g/d, resulting in 0 and 2,500 mg/d of 3-nitrooxypropanol, respectively. Both NOP and SiO_2, in powder form, were hand mixed with 80 g ground barley grain, 50 g wet molasses and 40 g canola oil to improve adhesion to feed particles and palatability. Cows were assigned to either treatment on d 1 of each period without incremental adaptation. To avoid contamination of feeding equipment at the farm, each treatment mixture was applied by hand-mixing into the TMR once daily, within 30 minutes of feeding. This protocol also simulated an on farm feeding scenario allowing for consumption of NOP throughout the day as feed is consumed.

2.2.3 Data and Sample Collection

The amount of feed offered and refused was recorded for individual cows at the time of feeding, and the amount of TMR fed was adjusted daily to maintain 5% refusals. The NOP content in refusals was not determined. Dietary ingredients were sampled (approximately 500 g) on d 25 to 27 and composited for each period to determine the chemical composition of the diet. All samples were dried for 72 h at 55°C in a forced air

oven and stored at 4°C until further analysis. Additionally, diets were adjusted weekly to maintain the same concentrate-to-forage ratio on a DM basis. Body weight was measured at the beginning and end of each period. Milk yield was recorded at every milking, and milk samples (approximately 50 mL) were taken from 6 consecutive milkings from d 25 to 27 and stored at 4°C with 2-bromo-2-nitropropane-1,3-diol until milk composition analysis.

Rumen digesta was collected through a rumen cannula, from five locations in the rumen (cranial dorsal, cranial ventral, central rumen, caudal dorsal and caudal ventral) before feeding at 0830 h on d 22 and 28. Digesta samples were collected by grabbing a handful of rumen digesta and transferring to a 50-mL sterile tube for each location. The tubes were immediately frozen on dry ice and stored at -80 °C until subsequent microbial analysis. A separate sample of rumen digesta was obtained at 0, 6 and 12 h after feeding on d 22 and 28. The rumen digesta were collected by grabbing a handful from the same five locations (approximately 100 mL from each location). These digesta samples were combined to one sample per cow per sampling time, and strained through two layers of perforated fabric (WeedBlock, Easy Gardener, Waco TX, USA) to obtain rumen fluid samples. Thus, a 15 mL sample of rumen fluid was obtained and placed on ice until centrifugation at 3, 000 × *g* for 20 min at 4ºC and subsequently stored at -20ºC. Rumen fluid samples were thawed and pooled to one sample per cow per period accounting for diurnal variation, and stored at -20 °C until subsequent VFA profile analysis.

Enteric methane emissions from individual cows were measured consecutively for 5 d (d 23-27) using the sulfur hexafluoride (SF_6) tracer gas technique (Johnson et al., 1994), as modified by McGinn et al. (2006), using halters and polyvinyl chloride neck

yokes (approximately 2 L internal capacity). The halters and yokes were designed to allow for a 50% reduction in yoke vacuum over 24 h by an in-line stainless steel capillary tube. Brass permeation tubes (12.5 × 40 mm) containing pure SF_6 were sourced from Dr. Iwaasa (Agriculture and Agri Food Canada, Swift Current, SK) and stored at 39 ºC for at least 3 months prior to the study start date to determine release rates. The mean release rates were 3.25 ± 0.41 and 3.20 ± 0.23 mg/d (mean ± SD) for Group 1 and 2, respectively. Permeation tubes were placed in the rumen of individual cows 1 week prior to the beginning of the study. One week prior to methane collection, cows were adapted to halters and yokes placed around the neck for methane emission measurement.

For methane emission measurement, air was evacuated from yokes and they were placed on each animal at 0700 h on d 23, and yokes and halters were replaced every 24 h until d 27. Halters were checked daily for line blockages using pure nitrogen (N_2). Yokes were pressurized with N_2 and allowed to sit for 1 h, to obtain representative samples, and three 20-mL subsamples were taken from each yoke with a syringe, and injected into 6.8-mL containers for further analysis. Following sampling, yokes were cleaned 3 times by pressurizing with N_2, sitting for 1 h, and depressurizing. Yokes were again pressurized and allowed to sit until the following day to check for leakages, and used again for sample collection if no leakages were confirmed. Background concentrations of methane and SF_6 in the animal facility were determined by suspending halters and yokes in front of and above cows during the sample collection period. Background yokes were treated in the same manner as yokes on cows but halters were not changed on a daily basis.

2.2.4 Sample Analysis

Dried feed samples were ground through a 1-mm screen with a Wiley mill (Thomas-Wiley, Philadelphia, PA) and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis. Dry matter was determined by drying samples at 135ºC for 2 h (AOAC, 2000; method 930.15) and OM was determined after 4-h combustion at 550ºC (AOAC, 2000; method 942.05). Crude protein concentration was determined by flash combustion with gas chromatography and thermal conductivity detection (AOAC, 2000; method 990.03). The NDF concentration was determined using sodium sulfite and amylase (Van Soest et al., 1991), and ADF was determined by boiling samples in an acidic solution followed by filtration (AOAC, 2000; method 973.18). Crude fat was determined using a Tecator Soxtec System HT 1043 extraction unit (Tecator, Eden Prairie, MN, USA) according to AOAC method 2003.05 (AOAC, 2006) and starch concentration was determined as described in Hall (2009). Milk samples were individually analyzed for concentrations of milk fat, crude protein, lactose, MUN and SCC contents at the Alberta Central Milk Testing Laboratory (Edmonton, Alberta, Canada; AOAC, 2002). Rumen fluid samples were centrifuged at 12,000 × *g* for 10 min and supernatant was collected and analyzed for VFA concentration by gas chromatography as described by Schlau et al. (2011).

Frozen rumen content (about 0.5-1g) was thawed on ice and processed for DNA extraction. The bead-beating method was used to extract total DNA from the rumen digesta using the protocol outlined by Guan et al. (2008). After extraction, the concentration and quality of DNA was measured at A260 and A280 using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Following DNA extraction, DNA samples (50 ng/uL) were used for estimation of total populations of bacteria and methanogenic Archaea in rumen digesta by measuring the copy numbers of 16s rDNA gene using qPCR. The primer pair U2 (forward, 5'-ACTCCTACGGGAGGCAG-3'; reverse, 5'-GACTACCAGGGTATCTAATCC-3'; Stevenson and Weimer, 2007) was used to detect the copy numbers of total bacteria and the primer pair uniMet (F, 5'-CCGGAGATGGAACCTGAGAC-3'; R, 5'-CGGTCTTGCCCAGCTCTTATTC-3'; Zhou et al., 2009) was used to detect the copy numbers of total methanogenic Archaea. All qPCR was performed with SYBR green chemistry (Fast SYBR green master mix; Applied Biosystems, Burlington, ON), using the StepOnePlus real-time PCR system (Applied Biosystems, Burlington, ON). The amplification program included a fast cycle and a melting curve section. The program used for total bacteria was 95°C for 5 min, followed by 40 cycles at 95°C for 20 s and 62°C for 30 s, while the program for total methanogenic Archaea was 95°C for 5 s for initial denaturation and then 40 cycles of 95°C for 10 s, followed by annealing/extension for 30 s at 60°C. The final melting curve detection of both microbes were the same, with 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s with fluorescence collection at 0.1°C intervals. The standard curves were constructed using a serial dilution of plasmid 16s rDNA from clones *Butyrivibrio hungatei* (Li et al., 2009) and *Methanobrevibacter* sp. strain AbM4 (Zhou et al., 2010) for total bacteria and methanogenic Archaea, respectively. The final copy numbers of 16s rDNA of targeted microbes, per gram of rumen digesta, were calculated as described by Chen et al. (2012). The initial copy numbers of the standard curve were calculated based on the formula $(NL \times A \times 10^{-9})/(660 \times n)$, where NL is the Avogadro constant (6.02×10^{23} molecules per mol), A is

the molecular weight of the molecule in standard, and n is the length of the amplicon (bp). The serial dilution were assigned from 10^{-3} to 10^{-8}.

Total protozoa were estimated by analyzing the total copy number of 18S rRNA genes with primer pair P-SSU-316F (5' -CTTGCCCTCYAATCGTWCT- 3'; Huws et al., 2009) and P-SSU-539R (5' –GCTTTCGWTGCTAGTGTATT-3') using q-PCR with SYBRGreen Chemistry. The standard curve was constructed using plasmid DNA containing a cloned sequence (223bp) using the same primer set that has been confirmed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches against *Entodinium longinucleatum* 18S rRNA gene (Accession number AB481099). The qPCR was performed using a fast cycle and a melting curve section. The program was 95°C for 5 s for initial denaturation and then 40 cycles of 95°C for 10 s, followed by annealing/extension for 30 s at 60°C. The final melting curve detection of protozoa was 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s with fluorescence collection at 0.1°C intervals. Similarly, the copy number of total protozoa 18S rRNA genes per gram of rumen digesta was calculated using the same formula as described for total bacteria and methanogenic Archaea.

Gas samples taken from the yokes were analyzed at the Lethbridge Research Centre (Lethbridge, AB, Canada) for concentrations of SF_6 and methane using gas chromatography (model 5890, Agilent Technologies, Santa Clara, CA) with flame-ionization detection for methane and electron capture detection for sulfur hexafluoride using the method described by Chung et al. (2011).The SF_6-to-methane ratio, corrected for background levels, was determined for each sample according to McGinn et al.

(2006). Daily methane emission data were averaged to report one value per cow per period, and data from cows with less than 2 days of successful measurements were excluded from statistical analysis.

2.2.5 Calculation and Statistical Analysis

Yield of 4% FCM was calculated according to the equation: 4% FCM = (milk yield, kg × 0.4) + (fat yield, kg × 15), and ECM yield was calculated by the equation as per Tyrell and Reid (1965): ECM = (milk yield, kg × 0.327) + (fat yield, kg × 12.95) + (protein yield, kg × 7.25). Feed efficiency was calculated as 4% FCM, kg divided by kg DMI. Energy expenditure to maintenance, milk production, change in BW and methane was calculated according to NRC (2001). Gross energy intake from feed was calculated using the formula: GE (Mcal/kg) = (0.0415 × carbohydrate, %) + (0.094 × fat, %) + (0.057 × protein, %) (NRC, 2001)

Data were analyzed using the MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) according to the following model:

$$Y_{ijkl} = \mu + P_i + T_j + R_k + C(R)_{l(k)} + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is overall mean, P_i is fixed effect of period, T_j is fixed effect of treatment, R_k is fixed effect of parity, $C(R)_{l(k)}$ is random effect of cow nested in parity, and e_{ijkl} is residual. Effects of group and parity × treatment interaction were originally included in the model, but removed from the final statistical model as their effects were not significant for primary response variables. Significance was declared when $P < 0.05$ and tendencies were discussed when $P < 0.10$.

2.3 Results

The success rate for methane emission measurement was 64%. Of the unsuccessful measurements, 92% were caused by damages to the halters or yokes and the remaining 8% was due to inadequate amounts of SF_6 in gas subsamples resulting in the inability to calculate methane emissions correctly. As the methane emission data with less than 2 d of successful measurements were excluded from statistical analysis, 5 data points were treated as missing values. Final n values were 8 and 11 for CON and NOP, respectively. Overall concentration of methane and SF_6 found in yokes placed on cows yielding a successful measurement was 36.8 ± 24.6 ppm and 49.2 ± 27.8 ppt (mean ± SD), respectively. In background yokes, concentrations of methane and SF_6 were 10.3 ± 4.6 ppm and 9.48 ± 5.92 ppt (mean ± SD), respectively. For successful measurements, coefficient of variation for daily methane emission data within cow was 23.6 and 22.7%, respectively for NOP and CON, and they did not differ ($P = 0.97$).

Feeding 2,500 mg/d of NOP did not affect DMI compared with CON (Figure 2-1A). Cows fed NOP had decreased methane emissions (132 vs. 372 g/d; SEM = 23.1; $P < 0.0001$), even when normalized for DMI (7.18 vs. 17.8 g/kg DMI; Figure 2-1B) or expressed as a percentage of gross energy intake (2.20 vs. 5.45%; SEM = 0.31; $P < 0.0005$) when compared to CON.

Milk yield and milk component concentration were not affected when cows were fed NOP compared to CON (Table 2-2). Furthermore, when milk yield was corrected for milk fat or energy content, there were no differences between treatments. However, BW gain was greater when cows were fed NOP compared to CON (1.06 vs. 0.39 kg/d; $P = 0.05$), but feed efficiency was not affected by treatment.

Feeding NOP did not affect total concentration of VFA; however, it reduced the molar proportion of acetate (52.1 vs. 55.5 mol/100 mol of VFA; $P = 0.04$; Table 2-3) and tended to increase the proportion of propionate (26.4 vs. 23.9 mol/100 mol of VFA; $P = 0.06$) when compared to CON. This resulted in a decreased acetate-to-propionate ratio when cows were fed NOP (2.02 vs. 2.36; $P = 0.04$) compared to CON. No treatment effects were observed for other VFA. Total methanogen copy numbers were reduced with feeding of NOP (0.95 vs. 2.69×10^8 / g of rumen digesta; $P = 0.001$) compared with CON, but total bacteria and protozoa copy numbers were not affected by treatment. There was a tendency for a positive relationship between methanogen count number and methane emissions normalized for DMI ($P = 0.08$; $y = 8.89 + 2.05 \times 10^8 x$, $r^2 = 0.16$).

2.4 Discussion

One of the primary objectives of this study was to determine the effects of NOP on methane emissions of lactating dairy cows without moving them to metabolic chambers. By using the SF_6 tracer gas technique, we found that feeding 2,500 mg/d of NOP to lactating dairy cows reduced methane emissions by 60% without a reduction in DMI. The observed reduction in methane emission is slightly lower than that reported in vitro (Romero Perez et al., 2013a) but far greater than previously observed when the compound was tested in vivo (Martinez-Fernandez et al., 2013; Reynolds et al., 2013). Romero Perez et al. (2013a) measured in vitro methane production using a rumen simulation technique and found that methane production was reduced by 75%. However, it is important to note that this reduction was seen in an in vitro system where 5 mg/d of NOP was added to 10 g/d of feedstuff compared to 2, 500 mg/d of NOP in 19.5 kg of DMI in an in vivo setting. In comparison to other in vivo studies, when 100 mg/d of NOP

was dosed directly in the rumen of sheep fed a 60% forage diet, methane emission was reduced by 23% (Martinez-Fernandez et al., 2013). Similarly, when 2,500 mg/d was dosed directly into the rumen of lactating dairy cattle, methane emission was reduced by 10% (Reynolds et al., 2013). When 2,720 mg/d was top-dressed on to a 60% forage TMR fed to beef heifers, methane emission was reduced by 33% (Romero Perez et al., 2013b).

All previous in vivo studies evaluating NOP were conducted using chambers to measure methane emissions, whereas the current study used the SF_6 tracer gas technique. However, greater reduction in methane emission from lactating dairy cows in the current study cannot be explained by difference in methodology alone because we used a crossover design in which each animal serves as their own control. Any potential bias or errors leading to less accurate methane measurement would therefore affect both NOP treatment and CON, and as such, would not affect the evaluation of the effects due to treatment. However, coefficient of variation for daily methane measurements using SF_6 tracer gas technique exceeded 20% in the current study suggesting that this technique would not be appropriate to evaluate minor treatment effects on methane emissions. It has been suggested that cannulated animals not be used with the SF_6 tracer gas technique due to an increase in variability (Beauchemin et al., 2012). However, we were able to detect significant treatment effects of NOP on methane emissions with SF_6 tracer gas technique partly because of the great magnitude of reduction. Despite the inherent technical problem, SF_6 tracer gas technique was considered advantageous for our study as it allowed for animals to stay in their normal environment, and treatment effects on DMI and animal performance were determined while methane emissions were being measured.

One possible explanation for a higher reduction in methane emissions in the current study, compared to previous in vivo trials, could be that the compound was mixed into the TMR. This approach might have allowed the cows to consume NOP throughout the day as feed was ingested compared with dosing into the rumen. When NOP was dosed into the rumen of dairy cattle, Reynolds et al. (2013) found that methane production dropped substantially immediately after dosing, but the effect was only sustained for one to two hours. It was hypothesised that this fast decrease in efficacy was likely linked to the very high solubility of the compound that would rapidly leave the rumen, particularly in high producing dairy cow with decreased rumen retention time. Additionally, the reduction in methane emission post dosing may coincide with the period that methanogens are expected to be highest. Leedle and Greenling (1988) found that when steers were fed a 25% forage diet, methanogen counts increased shortly after feeding, dropped and then continued to rise throughout the day (3.7×10^8 per g ruminal contents at 4 h vs. 8.3×10^8 per g ruminal content at 20 h, post feeding). Allowing for continual consumption of NOP over a 24-h period, as was done in the current study, could potentially lead to a sustained reduction of methane production throughout the day. As cows were fed TMR ad libitum, allowing for 5% refusal in the current study, actual NOP intake could have been lower than 2,500 mg/d for some animals. Although actual NOP intake and consistency of NOP supply to the animal were not determined in the current study, our data suggest that mixing the compound in TMR is more effective at reducing methane emissions compared with dosing into the rumen directly.

In the current study, there was a tendency for a positive relationship between number of methanogens and methane production (Figure 2-2); however, a direct cause-

and-effect relationship between methanogen abundance and methane production has not been confirmed in the literature. Contrary to our results, previous work by Martinez-Fernandez et al. (2013) found no difference in microbial profile in sheep dosed with NOP, and similarly, Romero Perez et al. (2013b) reported that total methanogen counts were not affected by NOP. In both cases, the diet fed to the animals was 60% forage (DM basis), and they reported less reduction in methane emissions due to NOP compared to the current study where a 38% forage diet was fed. Hook et al. (2011) showed that the species of methanogens present in the rumen was different for a high concentrate diet compared to a high forage diet fed to non-lactating dairy cattle while the level of concentrate did not affect total methanogen counts. Similarly, Zhou et al. (2010) found that regardless of dietary energy content, the total population of methanogens present in the rumen of steers was not different; however, the predominant species differed. These previous data indicate that efficacy of NOP in reducing methane emissions might be diet dependent and that NOP may affect specific species of methanogens that are present with low forage diets to a greater extent, which may partly explain greater animal responses to NOP in the current study compared with the other in vivo studies (Martinez-Fernandez et al., 2013; Reynolds et al., 2013; Romero Perez et al., 2013b).

In addition, ecto- and endosymbiotic relationships exist between protozoa and some methanogens, and their contribution is estimated at up to 37% of total methane production in the rumen (Dohme et al., 1999; Hegarty, 1999; McAllister and Newbold, 2008). There is often a relationship between methane production, methanogens and protozoa counts. In particular when effects of feeding lipids are assessed, a reduction in protozoa is often observed along with a reduction in methane (Dohme et al., 1999;

Beauchemin et al., 2009b). In the current study, there was no change in protozoa counts when NOP was fed and as such, the reduction in methanogens may not be attributed to changes in protozoa, but to the direct effects of NOP on methanogen counts or their function. The current data further support the suggestion that NOP reduces methane formation through impeding methanogenesis thus reducing methanogen counts. This effect could be diet dependent, therefore allowing for a specie specific response which would explain the discrepancy in results between the current study and other studies using NOP in vivo. As such, further research is warranted to determine whether abundance of specific methanogen species is affected when NOP is supplemented in the diet.

The decrease in acetate concentration, tendency towards increased propionate concentration in ruminal fluid, and resulting reduction in acetate-to-propionate ratio is consistent with the inverse relationship reported for methane and propionate (Mathison et al., 1998). Promoting propionate production in the rumen can reduce methane production as it acts as an alternate hydrogen sink (Kobayashi, 2012). Alternatively, a reduction in methane can cause an increase in propionate production, and the lower acetate-to-propionate ratio observed in the current study supports that propionate production acted at least partly as an alternate pathway for hydrogen. Similarly, other research has found a reduction in the acetate to propionate ratio when NOP is fed (3.89 vs. 4.91, Martinez-Fernandez et al., 2013; 2.74 vs. 2.93, Reynolds et al., 2013; 2.1 vs. 3.3, Romero Perez et al., 2013b), suggesting fermentation in the rumen shifted towards propionate production. The authors acknowledge that the tendency for an increase in propionate concentration for NOP compared to CON would not exclusively explain the metabolic fate of available

hydrogen that is expected to increase from a 60% reduction in methane emissions. In an in vitro system, Immig (1996) studied the effects of 2-bromoethanesulfonic acid, a structural analog to coenzyme M that blocks the final step of methane formation which is similar to the expected mode of action of NOP. They found that there was a discrepancy between hydrogen produced during fermentation and recovered from the system through end products when methane was reduced. Those results suggested that there were additional hydrogen sinks and through stoichiometric calculations, they determined that there was an accumulation of formate, a precursor for methanogenesis. As formate is readily metabolized in the rumen and difficult to measure in vivo (Hungate et al., 1970), further research is warranted to look at the effects of NOP on rumen fermentation pathways and alternate hydrogen sinks.

Contrary to our hypothesis, milk yield and milk component concentration were not affected despite a substantial reduction in methane; however, cows fed NOP gained more BW compared to CON (1.06 vs. 0.39 kg/d). When Reynolds et al. (2013) dosed 2,500 mg/d of NOP to mid lactation cows, FCM yield was also not affected and BW change was not reported. The results found in the current study could be due to the fact we used cows after peak lactation and it has been found that partitioning of ME intake towards milk energy output decreases as lactation stage proceeds while partitioning towards body tissue energy increases (Kirkland and Gordon, 2001).

To quantify the additional net energy provided by reduced methane emissions, energy outputs were calculated and compared between NOP and CON cows. When energy expenditures were calculated according to NRC (2001), net energy outputs towards maintenance (9.43 vs. 9.48 Mcal/d) and lactation (23.8 vs. 23.6 Mcal/d) were

similar between NOP and CON cows, respectively; however, NOP cows had more net energy output towards BW gain compared to CON cows (5.86 vs. 2.12 Mcal/d). Assuming that energy outputs for heat, urine and feces were not affected by treatment, the reduction in energy lost as methane (1.75 vs. 4.94 Mcal/d, respectively for CON and NOP) accounted for approximately 80% of the difference in total energy expenditures.

Given that cows used in this study were in mid-lactation and the TMR fed was formulated to exceed their nutrient requirements for lactation, it is reasonable that excess ME, due to a reduction in methane production, would go towards BW gain rather than lactation. As such, in future studies, it would be necessary to evaluate the effects of feeding NOP on milk production and energy partitioning of cows in early lactation. In moving forward with establishing the efficacy of feeding NOP to reduce methane emissions from ruminants, its effects on nutrient digestibility and metabolism should also be evaluated. In addition, the current study evaluated effects of NOP for relatively short periods (i.e., 28 d) using a crossover design, in which possible confounding carryover effects of treatment cannot be excluded. A long-term study is warranted to determine effects of feeding NOP on rumen fermentation and animal performance more accurately.

2.5 Conclusion

Feeding 2,500 mg/d NOP reduced enteric methane emissions from lactating dairy cows by 60% without affecting DMI, and the reduction in methane emission was associated with decreased methanogen counts and acetate-to-propionate ratio in the rumen. In addition, although milk production was not affected by treatment, cows fed NOP increased BW gain, indicating that the reduction in methane emissions increased energy availability to animals.

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Table 2-1. Ingredients and nutrition composition of experimental diet

Diet ingredients, %DM	
Barley silage	37.9
Dry ground corn	31.4
Canola meal	11.3
Corn gluten meal	8.4
Beet pulp	6.2
Canola oil	1.4
Limestone	1.2
Salt	1.2
Calcium diphosphate	0.6
Magnesium oxide	0.3
Mineral and vitamin premix[1]	0.1
Nutrient composition	
DM, %	53.5
OM, % DM	90.8
CP, % DM	19.6
NDF, % DM	26.5
ADF, % DM	18.4
Starch, % DM	26.8
Ether extracts, % DM	5.0

[1]Contained 17,413 KIU/kg vitamin A, 1,714 KIU/kg vitamin D_3, 57 KIU/kg vitamin E, 579 mg/kg Co, 28,586 mg/kg Cu, 1,286 mg/kg I, 51,429 mg/kg Mn, 85,714 mg/kg Zn, and 571 mg/kg Se.

Table 2-2. The effects of feeding 3-nitrooxypropanol (NOP) to lactating dairy cows on body weight change, milk yield and milk components

	Treatment[1]			
Variable	CON	NOP	SE	*P*
BW change, kg/d	0.39	1.06	0.21	0.05
Milk yield, kg/d	35.6	34.5	1.32	0.30
Milk fat[2], %	3.31	3.63	0.22	0.13
Milk crude protein, %	3.13	3.12	0.07	0.73
Milk lactose, %	4.65	4.65	0.03	0.98
4% FCM yield[2], kg/d	31.5	32.3	1.25	0.42
ECM yield[2], kg/d	34.6	35.0	1.20	0.56
Feed efficiency[2], 4% FCM/DMI	1.60	1.69	0.06	0.26

[1]Treatments: control (CON) = 25 g/d silicone dioxide; NOP = 2,500 mg/d 3-nitrooxypropanol n=12 for both CON and NOP unless otherwise noted

[2] One data point for milk fat content was removed due to being an outlier resulting in n = 12 and 11 for CON and NOP, respectively.

Table 2-3. The effects of feeding 3-nitrooxypropanol (NOP) to lactating dairy cows on volatile fatty acid profile of rumen fluid and bacterial profile counts

	Treatment[1]			
Variable	CON	NOP	SE	*P*
VFA				
Total, m*M*	103	109	5.29	0.39
Acetate, mol/100 mol of VFA	55.5	52.1	1.08	0.04
Propionate, mol/100mol of VFA	23.9	26.4	0.85	0.06
Butyrate, mol/100 mol of VFA	14.3	14.8	0.85	0.59
Isobutyrate, mol/100 mol of VFA	1.10	1.08	0.06	0.82
Valerate, mol/100 mol of VFA	2.25	2.57	0.19	0.27
Isovalerate, mol/100 mol of VFA	1.84	2.02	0.14	0.15
Caproate, mol/100 mol of VFA	1.17	1.06	0.25	0.62
Acetate-to-propionate ratio	2.36	2.02	0.15	0.04
Total bacteria copy numbers, $\times 10^{10}$/ g rumen digesta	34.5	20.5	5.62	0.11
Methanogen copy numbers, $\times 10^{8}$/ g rumen digesta	2.69	0.95	3.01	0.001
Protozoa copy numbers, $\times 10^{5}$/ g rumen digesta	1.69	1.35	0.25	0.25

[1]Treatments: control (CON) = 25 g/d silicone dioxide; NOP = 2,500 mg/d 3-nitrooxypropanol; n=12 for both CON and NOP

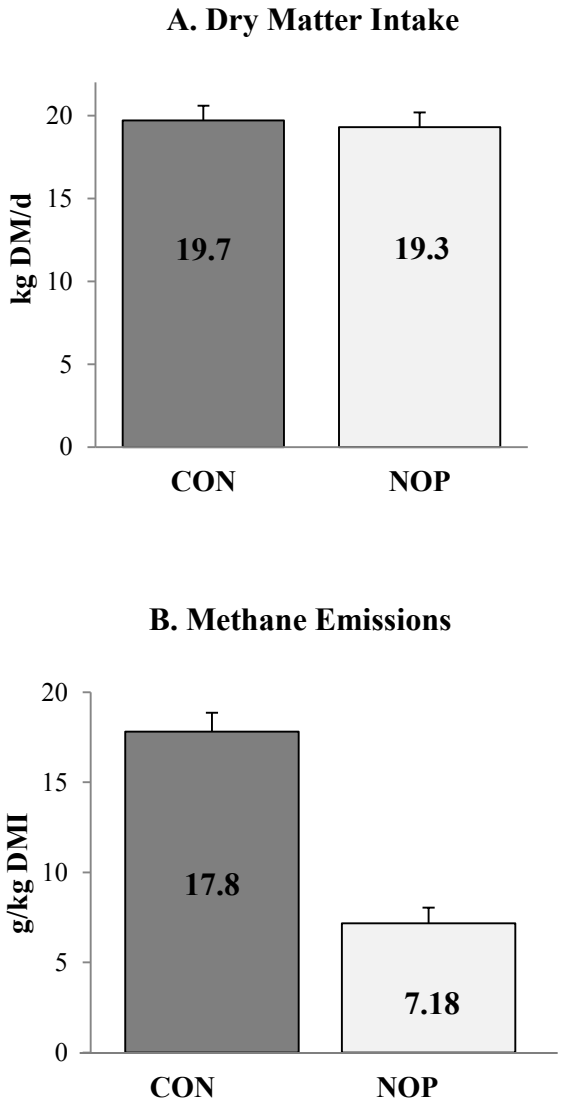

Figure 2-1: Effects of feeding 2,500 mg/d of 3-nitrooxypropanol (NOP) or 25 g/d silicone dioxide (CON) to lactating dairy cows on DMI (A; SEM = 0.66; $P = 0.36$; n = 12 for both CON and NOP) and methane emissions (B; SEM = 0.95; $P < 0.001$; n = 8 and n =11 for CON and NOP, respectively).

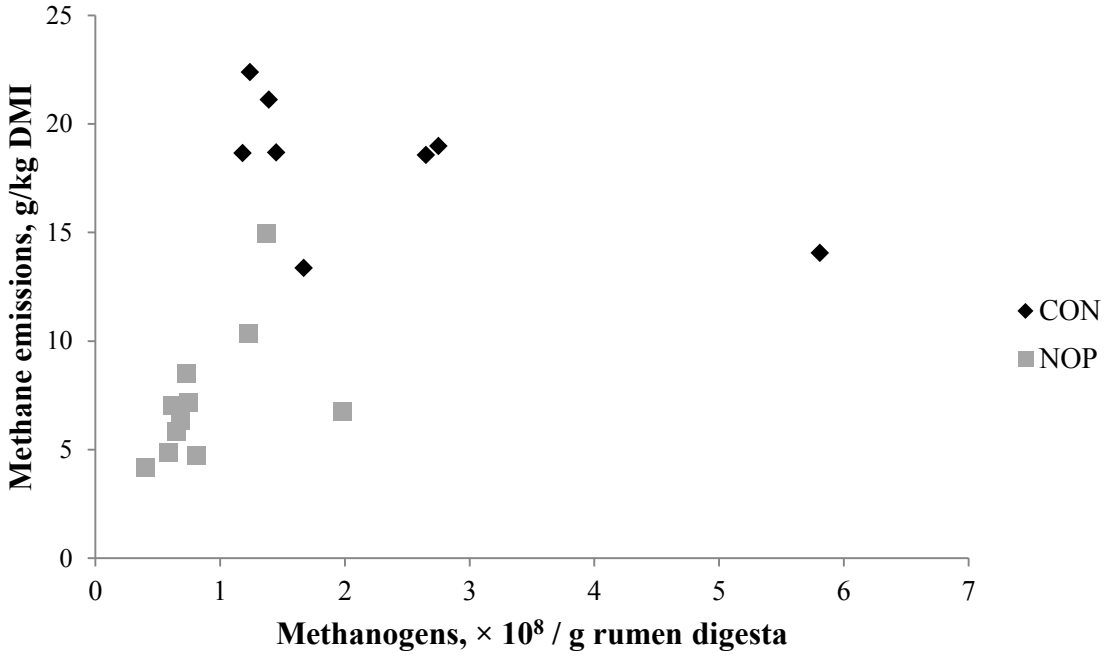

Figure 2-2: Relationship between methanogen counts and methane emission per kg of DMI when 2,500 mg/d of 3-nitrooxypropanol (NOP) or 25 g/d silicone dioxide (CON) was added to the diet of lactating dairy cows (n = 8 and 11 for CON and NOP, respectively).

A tendency was observed for the overall relationship between methanogens and methane emissions ($P = 0.08$; $y = 8.89 + 2.05 \times 10^8 x$, $r^2 = 0.16$).

3.0 STUDY 2: The effects of feeding 3-nitrooxypropanol, at varying levels, on methane emissions, animal performance, and nutrient metabolism of lactating Holstein cows

3.1 Introduction

Reducing methane emissions from ruminant agriculture has been a long time area of focus (Knapp et al., 2014) as awareness regarding greenhouse gases increases. While not only beneficial to the environment, inhibition of methane from ruminants comes with the potential for energy retention in the animal (Moss et al., 2000) as 2-12% of ingested energy can be lost to methane (Johnson and Johnson, 1995). Great efforts have gone towards reduction of methane emissions from ruminants with techniques varying from manipulation of the animal through genetic and managerial approaches, the diet through feed and nutritional management, or the rumen by feeding substances to directly or indirectly inhibit methanogenesis (Knapp et al., 2014; Eckard et al., 2010).

Methanogenesis acts as the major hydrogen sink in the rumen, and hydrogen balance in the rumen affects rumen fermentation and fermentation products (Hegarty and Gerdes, 1999; Janssen, 2010). Because of this, a reduction in methane production can lead to digestive upset resulting in reduced nutrient digestibility or negative impacts on animal performance. While not always the case, examples where this has occurred can be seen through feeding of lipids (Beauchemin et al., 2008) and tannins or saponins (Goel and Makkar, 2012).

Most recently, there has been great interest in developing synthetic compounds with direct inhibition on methane production through pathways specific to methanogenesis (Soliva et al., 2011; Duval and Kindermann, 2012) that do not alter

rumen fermentation negatively. One compound shown to be effective at inhibiting methane production is 3-nitrooxypropanol. 3-nitrooxypropanol was found to reduce methane emissions by 10 and 33% when 2, 500 mg/d was dosed directly into the rumen of lactating Holsteins (Reynolds et al., 2014) or 2, 720 mg/d was consumed once a day by beef heifers (Romero Perez et al., 2013), respectively.

However, previous work by Haisan et al. (2014) showed that there was a much greater effect on methane emissions when NOP was mixed into the TMR, compared with dosing into the rumen, allowing for continual consumption of the compound. Methane emissions from dairy cows were reduced by 60% when 2,500mg/d was fed in conjunction with a 38% forage diet. While previous work by Martinez-Fernandez et al. (2014) and Romero Perez et al. (2013) found no effect on digestibility when dosed into the rumen of sheep or fed to beef heifers, Reynolds et al. (2014) found a tendency for reduced digestibility with dosing of the compound directly into the rumen of dairy cows. Therefore, it is unknown what the effect on digestibility will be when 3-nitrooxypropanol is fed in the TMR and a great reduction in methane is seen.

While 3-nitrooxypropanol has been shown to effectively reduce methane emissions, to date there have been no studies completed with 3-nitrooxypropanol mixed into a lactating Holstein TMR while measuring the effects on nutrient digestibility, within a commercial feeding setting. In addition, while the extent of methane mitigation by 3-nitrooxypropanol has been variable, the efficacy and optimal inclusion rate of NOP is still unknown. The objectives of the current study are to determine the effects of 2 inclusion rates of 3-nitrooxypropanol mixed in the TMR of lactating Holstein cows, on methane emissions, rumen fermentation, nutrient digestibility, blood metabolites as well as animal

performance. It was hypothesized that feeding of 3-nitrooxypropanol to lactating cows would reduce methane emissions resulting in a positive animal response of increased milk production or body weight gain.

3.2 Materials and Methods

Use of 10% 3-nitrooxypropanol on silicon dioxide (NOP; DSM Nutritional Products, Ltd., Switzerland) in animal feed was pre-approved by the Veterinary Drugs Directorate Division (Health Canada, Ottawa, ON, Canada). Milk was discarded during the study and upon completion a 14-d withdrawal period was implemented as per their instruction. All procedures were pre-approved by the Animal Care and Use Committee for Livestock at the University of Alberta and conducted according to guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

3.2.1 Experimental Design, Diet and Treatment

Fifteen lactating Holstein cows fitted with rumen cannulas (Bar Diamond Inc., Parma, ID) were used in a replicated 3 × 3 Latin square (n = 5) design study with 28-d periods; for each period, after 20 d of diet adaptation, data and samples were collected for 8 d. Two squares consisted of 6 primiparous cows with pre-experiment milk production of 28 ± 3.3 kg/d and three squares consisted of 9 multiparous cows with a pre-experiment milk yield of 37 ± 4.3 kg/d. Pre-experiment BW and DIM (means ± SD) were 522 ± 62.6 kg and 156 ± 23 d for primiparous cows and 624 ± 42.8 kg and 177 ± 9 d for multiparous cows. As we had capability to measure methane emission for up to 6 cows at any given time, the experiment was conducted using 3 groups of cows (Group 1, six primiparous cows in Squares 1 and 2; Group 2, six multiparous cows in Squares 3 and 4; Group 3,

three multiparous cows in Square 5), and the whole study protocol was staggered by 7 d between groups.

All cows were fed a 60%-forage diet as a TMR (Table 3-1) ad libitum, allowing for 5% refusals throughout the study, and had free access to water. The diet was formulated to meet the nutrient requirements of a 650-kg cow producing 38 kg milk per day (NRC, 2001). Cows were fed one of three experimental treatments: 25 g/d NOP (HIGH), 12.5 g/d NOP and 12.5 g/d silicon dioxide (SiO_2) (LOW) or 25g SiO_2 as a control (CON), containing 2,500, 1,250 and 0 mg/d of 3-nitrooxypropanol, respectively. The compound mixture was prepared and fed once daily with TMR as described by Haisan et al. (2014) to simulate an on farm feeding scenario. Cows were housed individually in tie stalls and milked twice daily in their stalls at 0400 and 1600 h.

3.2.2 Data and Sample Collection

Feed offered and refused by individual cows was recorded daily. Silage and concentrate samples were taken weekly and used to adjust the TMR to maintain the same forage-to-concentrate ratio on a DM basis. On d 25-27, approximately 500 g of feed ingredients and feed refusals were sampled and stored at 4˚C. On d 28 the samples were composited to yield one sample per cow per period which was then dried for 72 h at 55˚C in a forced air oven and stored until further analysis.

BW was measured at the beginning and end of each experimental period. Milk production was recorded at every milking and milk samples (approximately 50 mL) were taken from 6 consecutive milkings from d 25 to d 27 and stored at 4˚C with 2-bromo-2-nitropane-1,3-diol until milk composition analysis.

Rumen digesta was collected on d 21 and 28 from five locations in the rumen (cranial dorsal, cranial ventral, central, caudal dorsal and caudal ventral) before feeding, as previously described by Haisan et al. (2014), into 50-mL sterile tubes. The tubes were immediately frozen on dry ice and stored at -80˚ until microbial analysis. A separate sample of rumen digesta, collected in the same manner, was obtained at 0, 6 and 12 h after feeding on d 21 and 28 from the same locations, combined to yield one sample per cow per sampling time and strained through 2 layers of perforated fabric (WeedBlock, Easy Gardener, Waco TX). A 15mL sample of rumen fluid was obtained and placed on ice until centrifugation at 3, 000 *g* for 20 min at 4˚C, and approximately 10 mL of supernatant was stored at -20˚C until further processing.

Rumen pH was determined every 30 s from d 23 to d 28 using the Lethbridge Research Centre Ruminal pH Measurement System (Dascor, Escondido, CA). The pH data loggers were calibrated using buffers at pH 4 and 7 on d 23 and d 28; any shift in millivolt reading from electrodes between the two days was assumed to be linear and millivolt readings were converted to pH units as described by Penner et al. (2006). A 10-mL blood sample was taken from the coccygeal vessel of cows every 18 h over a 72 h period from d 25 to d 27 (n = 4) into tubes containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ). Immediately after collection, samples were placed on ice until centrifugation at 3, 000 *g* for 20 min at 4˚C. Approximately 5mL of plasma was kept and stored at - 20˚C. Samples were later thawed and pooled to one sample per cow per period for blood metabolite analysis. Fecal samples (approximately 100 g) were collected every 9 h, over a 72 h period from d 25 to d 27 (n = 8), then composited to yield one sample per cow per period and dried in a forced air oven and stored until analysis.

Enteric methane emissions from individual cows were determined from d 23 to d 27 using the sulfur hexafluoride tracer gas technique with halters and polyvinyl chloride yokes as described by Haisan et al. (2014). Brass permeation tubes (12.5 × 40mm) containing pure sulphur hexafluoride were placed in the rumen at least 20 d prior to methane collection. Mean release rates of sulphur hexafluoride from permeation tubes were (mean ± SD) 3.17 ± 0.35 mg/d, 3.57 ± 0.10 mg/d and 3.00 ± 0.21 mg/d for Groups 1, 2 and 3, respectively. Halters and yokes were placed on each animal at 0700 h daily and replaced every 24 h. Background concentrations of methane and sulphur hexafluoride in the barn were determined by hanging yokes in front of, and above cows during the sample collection period, and yokes were sampled in triplicate.

3.2.3 Sample Analysis

Feed ingredient, feed refusal and fecal samples were ground with a Wiley mill, through a 1-mm screen, (Thomas-Wiley, Philadelphia, PA). Dry matter concentration was determined after drying at 135˚ C for 2 h (AOAC, 2002; method 930.15), and OM concentration was subsequently determined by combustion at 600˚C for 2 h. Crude protein concentration (N × 6.25) was determined using a Leco FP-2000 N Analyzer (Leco Instruments Inc., St Joseph, MI). NDF concentration was determined using sodium sulfite and amylase (Van Soest et al., 1991) and indigestible NDF was determined after a120 h incubation in the rumen of a dry cow (Cochran et al., 1986) and used as a marker for apparent total-tract digestibility. Ether extract was determined using a Goldfisch extraction apparatus (Labconco, Kansas City, MO).

Milk samples were individually analyzed for concentrations of milk fat, crude protein, lactose and milk urea nitrogen at the Alberta Central Milk Testing Laboratory

(Edmonton, Alberta, Canada; AOAC, 2002). Total populations of bacteria, protozoa and methanogens in rumen digesta were estimated from a 1 g sample using q-PCR as previously described by Haisan et al., (2014). Rumen fluid samples were thawed and composited to one sample per cow per period, centrifuged at 12,000 × *g* and supernatant was analyzed for VFA concentration by gas chromatography as described by Schlau et al. (2011). Rumen NH_3-N concentration was determined as described by Fawcett and Scott (1960).

Plasma samples were analyzed for glucose concentration using a glucose oxidase/peroxidase enzyme and dianisidine dihydrochloride (Sigma, St. Louis. MO), and absorbance at 450 nm was determined using a SpectraMax 190 plate reader (Molecular Devices Corp., Sunnyvale, CA). Plasma beta-hydroxybutyrate (BHBA) concentration was determined after oxidation of BHBA to acetoacetate using 3-hydrooxybutyrate dehydrogenase (Roche, Mississauga, ON, Canada) and measuring reduction of NAD^+ to NADH using a SpectraMax 190 plate reader at 340 nm. Non-esterified fatty acids (NEFA) concentration was determined using a commercial kit (Wako Chemicals USA Inc. Richmond, VA) as was the concentration of insulin (Coat-a-Count Kit Diagnostics Products Corporation, Los Angeles, CA). Plasma urea N (PUN) was determined enzymatically as described by Fawcett and Scott (1960).

Gas samples taken from the yokes were analyzed at the Lethbridge Research Centre (Lethbridge, AB, Canada) for concentration of sulfur hexafluoride and methane using gas chromatography as described by Haisan et al. (2014). Daily methane emission data were averaged to report one value per cow per period. Daily minimum, mean and

maximum pH, as well as the duration and area that pH was below 5.8 were determined as described by Penner et al. (2006).

3.2.4 Calculations and Statistical Analysis

Milk composition was determined by accounting for different milk yields between morning and evening milkings. Milk yield was corrected for 4% milk fat using: 4% FCM= (milk yield, kg × 0.4) + (fat yield, kg × 15), energy corrected milk yield was calculated according to Tyrrell and Reid (1965): ECM = (milk yield, kg × 0.327) + (fat yield, kg × 12.95) + (protein yield, kg × 7.25). Feed efficiency was calculated as 4% FCM, kg divided by kg DMI.

Data were analyzed using the mixed procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC) according to the following model:

$$Y_{ijkl} = \mu + P_i + T_j + R_k + C(R)_{l(k)} + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is overall mean, P_i is fixed effect of period, T_j is fixed effect of treatment, R_k is fixed effect of parity, $C(R)_{l(k)}$ is random effect of cow nested in parity, and e_{ijkl} is residual. Effects of group and parity × treatment interaction were originally evaluated, but removed from the final statistical model as their effects were not significant for primary response variables. Significance was declared when $P < 0.05$ and tendencies were discussed when $P < 0.10$.

3.3 Results

During the study, one cow became ill for unknown reasons and had extremely low DMI (9.6 kg/d; 3.4 × SD lower than mean DMI of all cows). As a result, data were removed for that period thus resulting in n values of 15, 14 and 15 for CON, LOW and HIGH, respectively unless otherwise noted. The success rate for daily methane

measurement was 56% and of unsuccessful measurements, 51% were due to damages to halters or yokes and 49% due to inadequate amounts of SF_6 in gas subsamples resulting in the inability to calculate methane emissions accurately. The animal facility used has mangers where cows must put their heads under bars to eat which led to a majority of the damages. Given that a tie stall facility was used, cows were contained in stalls by neck collars which also interfered with the halters and yokes. In addition, while attempts were made to house the animals in a location within the barn with adequate air movement, ventilation was poor which resulted in varied levels of SF_6 in background yokes and yokes worn by cows. Therefore, methane data was calculated as an average of successful measurements for each cow, each period, and final numbers of methane emission data were 14, 12 and 12 for CON, LOW and HIGH, respectively.

3.3.1 Dry matter intake and methane

Dry matter intake did not differ between treatments ($P = 0.57$); however, methane was reduced with feeding of 3-nitrooxypropanol at both levels (Table 3-2). Feeding 1,250 mg/d reduced methane from 19.9 to 15.3 g/kg DMI and methane emission was 12.6 g/kg DMI when 2,500 mg/d was fed (Table 3-2). No difference in methane emissions (g/d, g/kg DMI and % GEI) was detected between the LOW and HIGH dose.

3.3.2 Body weight and milk production

Feeding of 3-nitrooxypropanol at both levels, (LOW and HIGH), did not have effects on change in BW ($P = 0.28$), milk yield ($P = 0.32$) or milk component concentration (Table 3-3), compared with the control. When milk yield was corrected for both fat and energy no treatment effects were seen. There was also no change in feed efficiency when the compound was fed.

3.3.3 VFA and bacterial profile

Overall the concentration of total VFA was not affected by treatment ($P = 0.42$); however, there was a reduction in molar proportion of acetate in a dose dependent manner ($P < 0.01$; Table 3-4) and an increase in propionate with feeding of the compound with no difference between the LOW and HIGH treatment. As such, the acetate-to-propionate ratio was reduced when the compound was fed ($P < 0.01$). There was no effect of NOP on rumen ammonia nitrogen concentration ($P = 0.24$). Total bacteria counts as well as protozoa and methanogen counts were also not affected by treatment ($P = 0.49$, $P = 0.64$, $P = 0.49$, respectively).

3.3.4 Rumen pH

There was no change in pH observed with feeding 1,250 or 2,500 mg/d 3-nitrooxypropanol and no change in duration of acidosis (defined as pH < 5.8) or severity of acidosis (Table 3-5).

3.3.5 Blood metabolites

There was no change in insulin ($P = 0.23$), NEFA ($P = 0.42$), BHBA ($P = 0.87$) or PUN ($P = 0.97$) between treatment groups (Table 3- 6); however, there was a tendency for increased glucose levels with feeding of 3-nitrooxypropanol ($P = 0.06$).

3.3.6 Digestibility

Feeding 2,500 mg/d 3-nitrooxypropanol increased digestibility of DM and NDF compared to CON; however, there was no difference between CON and LOW or LOW and HIGH (Table 3-7). There were tendencies for increased digestibility of organic matter, crude protein and ether extract.

3.4 Discussion

3.4.1 Methane Mitigation

Feeding NOP at both LOW and HIGH levels, in the TMR of lactating dairy cows, reduced methane production (g/d and g/kg DMI), without any change in DMI, which is in agreement with what was observed when NOP was dosed into the rumen of dairy cows (Reynolds et al., 2014). However, in the current study, there were no detectable differences in methane production between the LOW and HIGH dose. This is most likely due to the high SEM (i.e., 26.6 g/d) as Reynolds et al. (2014) was able to detect a difference of 28 g CH_4/d between treatments using chambers with a SEM of 11.0 g/d while in the current study a difference of 44 g CH_4/d was not detected with statistical significance.

Reynolds et al. (2014) used six late-lactation Holstein-Friesian cows and dosed 500 or 2,500 mg/d NOP into the rumen at feeding. When measured using respiration chambers, methane was reduced by 6.6% and 9.8% for 500 and 2,500 mg/d NOP, respectively; which is much less than the 23 and 37% reductions (LOW and HIGH, respectively) detected in the current study. It is unlikely that the difference in the extent of methane mitigation between Reynolds et al. (2014) and the current study is due to dietary effects. In both studies, cows were fed a diet with similar dietary characteristics and rumen pH was similar. However, as speculated by Haisan et al. (2014), differences in feeding method (i.e., mixed in TMR vs. directly dosed into the rumen) could be the reason as Reynolds et al. (2014) found a short term inhibitory effect on methane production that was sustained for 2-3 hours when NOP was dosed into the rumen. In the current study, continual consumption of NOP throughout the day would allow for

continuous effects on methane emissions thus resulting in a lower level of emissions overall.

The extent to which methane emissions were reduced was less in the current study than previously reported by Haisan et al. (2014) where methane emissions were reduced by 60% when NOP was fed at 2, 500 mg/d mixed in a 38%-forage TMR. There are several factors that could be the reason for this discrepancy, starting with type of diet fed. In the study by Haisan et al. (2014), a low forage diet was fed and while pH was not measured, the diet would be associated with a lower rumen pH. Methanogens have been found to be sensitive to low rumen pH (Hook et al., 2010); in the previous study (Haisan et al., 2014), the NOP treatment decreased methanogen copy number per gram of digesta. However, in the current study, there was no effect of feeding NOP on rumen pH or total bacteria, methanogen or protozoa copy numbers. This would suggest there was a synergistic effect of rumen pH and NOP to reduce methane emissions, and methanogen count that was not seen in the current study.

It is important to note that 10 of 15 cows were used in the previous study (Haisan et al., 2014), and microbial adaptation to NOP might be possible for those cows, resulting in a weaker response to NOP in the current study. However, the effect of period was not significant for primary response variables in either study, indicating that microbial adaptation to NOP would not likely explain the reduced treatment effects on methane emissions for the current study compared to the previous study (Haisan et al., 2014). Further research should be conducted to investigate the potential synergistic effects of rumen pH and NOP.

3.4.2 Rumen fermentation and digestibility

Reynolds et al. (2014) found a tendency for reduced digestion of DM, OM, CP and ADF with dosing of 2,500 mg/d NOP compared with CON. As methanogenesis is the main hydrogen utilizing pathway in the rumen, a reduction in methanogenesis is often associated with an accumulation of hydrogen acting as a feedback inhibitor (Immig, 1996), having detrimental effects on OM digestion and microbial growth (Mathison et al., 1998) in *in vitro* systems. In the previous study by Haisan et al. (2014) and the current study, an increase in propionate does not fully account for the metabolic fate of additional hydrogen from methane reduction. As reported by Reynolds et al. (2014), accumulation of hydrogen could have negatively affected nutrient digestibility. However, Martinez-Fernandez et al. (2014) and Romero Perez et al. (2013) found no reduction in DM degradation when NOP was provided to sheep and beef cattle, respectively. As discussed in detail by Janssen (2010), older literature reported that a reduction in methane emissions due to dietary management was accompanied by an increase in hydrogen concentration. More recently, van Zijderveld et al. (2011) reported an increase in hydrogen production when methane emissions were reduced by 16% using nitrate without any effect on nutrient digestibility. However, it is important to remember that hydrogen was measured in air, meaning that hydrogen may not accumulate in the rumen, but is rather expelled from the animal via respiration or eructation without negative effects on digestibility.

In the current study, an increase in DM and NDF digestibility and tendencies for increased digestibility of OM, CP and ether extract were observed. These results were not expected, and we cannot explain why feeding NOP increased nutrient digestibility.

Nonetheless, the current study provided no evidence suggesting negative effects of NOP on nutrient digestibility. In the current study, rumen pH and total VFA concentration were not affected by NOP at both feeding levels, and these results suggest that hydrogen may not have built up to the extent to impede fermentation in the rumen, but likely released from the rumen.

3.4.3 Energy Utilization

Methane emissions were reduced with feeding of 2,500 mg/d compared to the CON (4.0 vs. 6.2% of gross energy intake). Despite the reduction in measured methane emission and energy output in methane (5.01, 3.64, and 3.07 Mcal/d for CON, LOW and HIGH, respectively; $P < 0.01$), NOP treatment did not increase milk yield or BW gain in the current study. Total net energy expenditure for maintenance, lactation and body weight did not differ among treatments (30.5, 31.2, and 32.1 Mcal/d for CON, LOW and HIGH, respectively; $P = 0.67$). It should be noted that numerical decreases in milk production and numerical increases in BW for LOW and HIGH treatments compared with CON resulted in similar total energy outputs for all treatments.

In the previous study (Haisan et al., 2014), an increase in BW gain accounted for 80% of the difference in energy loss to methane assuming energy outputs to heat, urine and feces were the same. However, this did not hold true in the current study. Gross energy expenditure includes not only that used for maintenance and animal performance but energy loss to feces, urine and gasses. An increase in energy lost to feces is associated with an increase in nutrients found in the feces which was not the case in the current study. Energy lost to urine has a direct relationship to nitrogenous compounds present in urine and there is a positive correlation between urine nitrogen and urine energy (Street et

al., 1964). Plasma urea nitrogen is shown as a strong indicator of urinary-N loss (Kohn et al., 2005), as well as milk urea nitrogen. In the current study, there was no change in either plasma or milk urea nitrogen, indicating that urinary-N excretion did not likely increase. Therefore while not directly measured in the current study, it can be inferred that urinary-N losses were not increased with feeding of NOP. If hydrogen production is increased with feeding of NOP, this partly accounts for unidentified loss of energy as hydrogen is an energy dense substance (0.034 Mcal/d of H_2; Afeefy et al., 2011; van Zijderveld et al., 2011). However, van Zijderveld et al. (2011) measured hydrogen production and calculated an increase of 0.9 g of hydrogen per day when methane emission was reduced by 16% and found the amount of energy that could be lost to hydrogen to be negligible at approximately 0.03 Mcal/day. While hydrogen was not measured in the current study, it is possible that hydrogen production accounted for some gaseous energy losses. Additionally, in the current study, there is no data for energy loss in heat, but it is possible that losses to heat were increased. Reynolds et al. (2014) reported that feeding NOP increased heat production expressed as a percentage of digested energy. While the exact mechanism is not known, with increased NDF digestibility for HIGH, it is possible that heat associated with fermentation increased with feeding of NOP.

In the three studies conducted with dairy cows, cows have been past peak lactation which could underestimate the effects of NOP on long-term lactational performance as cows were not in a negative energy balance. Studies conducted in early lactation, or for an entire lactation would provide a better understanding as to the relationship between reduced methane emissions and increased energy availability to

cows. In addition, conducting studies assessing the exact mode of action of NOP and how rumen fermentation, in terms of reduced end products such as hydrogen, is affected could provide insight into nutrient digestibility.

3.5 Conclusion

Feeding 1,250 and 2,500 mg/d of 3-nitrooxypropanol into the TMR of lactating cows reduced methane emissions by 23 and 37%, respectively without any negative effects on DMI or apparent total tract nutrient digestibility. Despite this, milk production and body weight gain were not affected by treatment, indicating that the reduction in methane did not increase the energy availability to animals.

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Table 3-1. Ingredients and nutrition composition of experimental diet

Diet ingredients, %DM	
Barley silage	60
Dry ground corn	20.2
Canola meal	7.3
Corn gluten meal	5.4
Beet pulp	4
Canola oil	0.9
Limestone	0.8
Salt	0.8
Calcium diphosphate	0.4
Magnesium oxide	0.2
Mineral and vitamin premix[1]	0.1
Nutrient composition	
DM, %	46.1
OM, %DM	90.7
CP, %DM	19.6
NDF, %DM	33.8
EE, %DM	3.4

[1]Contained 17,413 KIU/kg vitamin A, 1,714 KIU/kg vitamin D_3, 57 KIU/kg vitamin E, 579 mg/kg Co, 28,586 mg/kg Cu, 1,286 mg/kg I, 51,429 mg/kg Mn, 85,714 mg/kg Zn, and 571 mg/kg Se.

Table 3-2. Dry matter intake and methane emissions of cows fed CON, LOW or HIGH levels of 3-nitrooxypropanol

	Treatment[1]				
Variable	CON	LOW	HIGH	SE	P-value
DMI, kg/d	19.2	18.3	18.9	0.62	0.57
CH_4, L/min[2]	0.39[a]	0.29[b]	0.24[b]	0.03	<0.01
CH_4, g/d[2]	378[a]	275[b]	231[b]	26.6	<0.01
CH_4, g/kg DMI[2]	19.9[a]	15.3[b]	12.6[b]	1.62	0.01
CH_4, % GEI	6.2[a]	4.8[b]	4.0[b]	0.72	<0.01

[1]Treatments: control (CON) = 25 g/d silicone dioxide; LOW = 12.5 g/d silicone dioxide and 12.5 g/d 10% 3-nitrooxypropanol; HIGH = 25 g/d 10% 3-nitrooxypropanol

[2] n= 14, 12 and 12 for CON, LOW and HIGH, respectively

Table 3-3. Body weight and milk yield of cows fed CON, LOW or HIGH levels of 3-nitrooxypropanol

	Treatment[1]				
Variable	CON	LOW	HIGH	SE	P
Change in BW, kg/d	0.52	0.74	0.90	0.17	0.28
Milk yield, kg/d	27.7	26.2	26.3	0.99	0.32
Milk fat, %	3.44	3.46	3.47	0.10	0.94
Milk crude protein, %	3.17	3.14	3.19	0.05	0.81
Milk lactose, %	4.55	4.60	4.57	0.05	0.79
Milk urea nitrogen, mg/dL	13.0	13.4	13.5	0.49	0.74
4% FCM yield, kg/d	25.3	23.9	24.4	1.01	0.59
ECM yield, kg/d	27.7	26.1	26.6	1.06	0.53
Feed efficiency, 4% FCM/DMI	1.34	1.31	1.30	0.05	0.88

[1] Treatments: control (CON) = 25g/d silicone dioxide; LOW = 12.5 g/d silicone dioxide and 12.5 g/d 10% 3-nitrooxypropanol; HIGH= 25 g/d 10% 3-nitrooxypropanol

Table 3-4. Effects of feeding varying levels of 3-nitrooxypropanol on volatile fatty acid (VFA) profile, rumen ammonia nitrogen and microbial profile

	Treatment[1]				
Variable	CON	LOW	HIGH	SE	P-value
VFA					
Total, mM [2]	96.9	95.6	91.5	3.18	0.45
Acetate, mol/100mol of VFA[2]	58.4[a]	54.7[b]	52.6[c]	0.65	<0.01
Propionate, mol/100 mol of VFA[2]	22.0[b]	23.8[a]	24.4[a]	0.54	<0.01
Butyrate, mol/100 mol of VFA[2]	13.8[c]	14.9[b]	15.9[a]	0.28	<0.01
Isobutyrate, mol/100 mol of VFA[2]	1.16	1.19	1.18	0.05	0.93
Valerate, mol/100 mol of VFA[2]	1.84[c]	2.05[b]	2.25[a]	0.06	<0.01
Isovalerate, mol/100 mol of VFA[2]	2.06[b]	2.58[a]	2.57[a]	0.13	0.01
Caproate, mol/100 mol of VFA[2]	0.68[b]	0.74[b]	1.09[a]	0.06	<0.01
Acetate-to-propionate ratio[2]	2.67[a]	2.33[b]	2.17[b]	0.08	<0.01
NH_3-N, mg/dL	6.02	5.09	4.48	0.65	0.23
Total bacteria counts, $\times 10^{10}$	8.64	8.63	7.13	1.03	0.49
Methanogen counts, $\times 10^8$	2.78	2.72	2.47	0.25	0.64
Protozoa counts, $\times 10^5$	1.46	1.73	1.48	0.19	0.49

[1] Treatments: control (CON) = 25g/d silicone dioxide; LOW = 12.5 g/d silicone dioxide and 12.5 g/d 10% 3-nitrooxypropanol; HIGH= 25 g/d 10% 3-nitrooxypropanol

[2] n= 14, 13 and 15 for CON, LOW and HIGH, respectively

Table 3-5. Rumen pH for cows fed CON, LOW or HIGH levels of 3-nitrooxypropanol

	Treatment[1]				
Variable	CON	LOW	HIGH	SE	P-value
Ruminal pH					
Minimum	5.56	5.70	5.67	0.07	0.31
Mean	6.46	6.47	6.50	0.04	0.76
Maximum	7.14	7.08	7.12	0.03	0.41
Ruminal pH <5.8					
Duration, min/d	42.6	74.6	47.1	24.0	0.60
Area, pH × min/d	8.54	13.6	10.2	5.30	0.79

[1] Treatments: control (CON) = 25g/d silicone dioxide; LOW = 12.5 g/d silicone dioxide and 12.5 g/d 10% 3-nitrooxypropanol; HIGH= 25 g/d 10% 3-nitrooxypropanol

Table 3-6. Plasma metabolites and hormones for cows fed CON, LOW or HIGH levels of 3-nitrooxypropanol

	Treatment[1]				
Variable	CON	LOW	HIGH	SE	P-value
Glucose, mg/dL[2]	55.4	55.8	59.9	1.53	0.07
Insulin, µIU/mL	6.63	8.86	7.58	0.94	0.25
NEFA, µEq/L[3]	66.6	70.6	65.5	6.90	0.86
BHBA, mg/dL	10.2	10.0	10.3	0.40	0.86
PUN[4], mg/dL	9.55	9.45	9.41	0.62	0.98

[1] Treatments: control (CON) = 25g/d silicone dioxide; LOW = 12.5 g/d silicone dioxide and 12.5 g/d 10% 3-nitrooxypropanol; HIGH= 25 g/d 10% 3-nitrooxypropanol

[2] n= 14, 14 and 15 for CON, LOW and HIGH, respectively

[3] n= 13, 12 and 14 for CON, LOW and HIGH, respectively

[4] Plasma urea N

Table 3-7. Apparent total tract digestibility for cows fed CON, LOW or HIGH levels of 3-nitrooxypropanol

	Treatment[1]				
Digestibility, %	CON	LOW	HIGH	SE	P
Dry matter	58.4[a]	59.9[ab]	62.7[b]	1.23	0.05
Organic matter	62.0	63.5	65.8	1.15	0.07
Crude protein	61.2	61.9	65.3	1.43	0.10
Ether extract	85.4	83.4	86.1	0.88	0.10
Neutral detergent fibre	30.7[b]	33.7[ab]	38.0[a]	1.85	0.02

[1] Treatments: control (CON) = 25g/d silicone dioxide; LOW = 12.5 g/d silicone dioxide and 12.5 g/d 10% 3-nitrooxypropanol; HIGH= 25 g/d 10% 3-nitrooxypropanol

4.0 General Discussion

4.1 Summary of research findings

Previous research had shown that NOP was effective at reducing methane *in vitro* (Romero-Perez et al., 2013), and when dosed into the rumen of sheep (Martinez-Fernandez et al., 2014) or dairy cattle (Reynolds et al., 2014) or fed once daily to beef cattle (Romero-Perez et al., 2013). However, no studies had been conducted with inclusion of NOP in the TMR of lactating Holstein cows while maintained in a commercial environment. As such, the sulfur hexafluoride tracer gas technique was used to measure methane emissions in both studies.

Study 1 evaluated the effects of feeding 3-nitrooxypropanol (NOP), at 0 mg/d (CON) and 2,500 mg/d (HIGH), mixed into a 38% forage TMR on methane emissions, animal productivity and rumen fermentation. In Study 1, methane emissions were reduced by 60% without affecting DMI. While there was no change in milk production, body weight gain was significantly greater for cows fed NOP. In addition, concentration of total VFA did not change, but there was a reduction in acetate and tendency for increase in propionate proportion. NOP did not affect protozoa counts, but there was a reduction in methanogen copy count number.

The objectives of Study 2 were similar to those of Study 1. However, NOP was included at two different levels, 1,250 mg/d (LOW) and 2,500mg/d (HIGH) in a 60% forage diet. Methane emissions, animal productivity, and rumen fermentation were measured, with the addition of rumen pH, nutrient digestibility and blood metabolites. In Study 2, methane emissions were reduced 23 and 37%, for the LOW and HIGH treatment,

respectively, when compared to CON, with no impact on DMI. Milk production, milk components or body weight gain were not affected by treatment. The total VFA concentration in rumen fluid did not change, but a reduction of molar proportion of acetate in a dose dependent manner and an increase in molar proportion of propionate were observed. In Study 2, there was no change in total bacteria, protozoa or methanogen counts, and no change in rumen pH. Feeding NOP tended to increase plasma glucose concentration, but did not affect the other metabolites evaluated. Feeding the HIGH dose of NOP increased apparent total tract digestibility of DM and NDF and there was a tendency for increased digestibility of organic matter, crude protein and crude fat.

4.2 Use of the SF_6 tracer gas technique

The SF_6 tracer gas technique was used in both studies to simulate an on farm scenario as closely as possible. A criticism of using chambers for methane measurement is that they remove the animal from their environment, which can cause changes to animal behavior and feed intake. In order to minimize these negative effects, SF_6 was used. However, use of SF_6 with cannulated animals has been criticised by many because of the potential for gaseous losses from the cannula. In order to address these concerns, both studies were designed in a manor such that any discrepancies would be carried throughout. By using a crossover and latin square design study, each cow acts as its own control, therefore any problems associated with use of cannulated cows, such as possible leakage, are carried throughout the study. In addition, all cows had tight fitting cannulas as recommended by Beauchemin et al. (2012).

In Study 1, the success rate for methane measurement was 64%, and 92% of unsuccessful measurements were caused by damages to the halters and yokes or blockages in halters. The SF_6 technique was primarily designed to measure emissions from animals in a grazing situation with only minor physical obstructions that could damage the system (ie. nothing for animals to hit halters and yokes against). Unfortunately, the design of the animal facility involves mangers such that the cows must put their head under a bar. Because the yokes used had to be worn around the neck, they were hit against the bar when cows ate which led to damages to the yokes and halters. In addition, the diet fed contained small particles that were able to get into the tubing of the halters resulting in blockages and inaccurate air samples if blockages occur. In an attempt to overcome the challenge of blocked tubing in Study 2, mesh screen was added to the ends of the tubing at the nostrils of animals to help prevent feed particles from entering the tube.

In Study 2, the success rate for methane measurement was 56%, and 51% of unsuccessful measurements were due to damages while 49% was due to inadequate amounts of SF_6 in gas subsamples. Damages due to blocked lines was reduced, however physical damage to yokes was not overcome. In Study 2, cows were housed at the end of the barn next to overhead doors in an attempt to improve air flow. However, inadequate amounts of SF_6 in gas subsamples were still likely due to poor ventilation in the animal facility thus resulting in higher SF_6 background concentrations (Beauchemin et al., 2012). While this was not a major problem in Study 1, Study 1 was conducted between May-July when air quality and movement is better in the facility, compared to July-October when Study 2 was conducted.

In some cases, little SF_6 was detected in air samples collected from cows, and in these cases, permeation tubes were removed from the rumen and replaced with a different permeation tube. It was found that removed permeation tubes had material accumulated in the nut where SF_6 would normally be released from the tube which could have contributed to lower than normal levels detected. At the end of the experiment, all permeation tubes were removed with some having blocked ends and others not with no apparent reasons for this discrepancy.

Overall use of the SF_6 technique served its purpose; however, with such low success rates, it would not be recommended for use in the animal facility again without modifications. In some cases, the technique has been used where animals wear yokes on their backs rather than necks. This adjustment would overcome some issues found in the two animal studies in regards to damages to the yokes. In addition, due to great variability in measurements, it would be unsuitable for use when small differences in methane emissions are expected between treatments. In Study 1, this was not an issue as a large reduction was seen, however in Study 2, a difference between LOW and HIGH in methane emission was not significant despite a 5% difference in methane emissions.

4.3 Questions to be addressed in future studies

Across all studies conducted using 3-nitrooxypropanol, methane emissions have been reduced without negative impacts on dry matter intake (Romero-Perez et al., 2013; Haisan et al., 2014; Martinez-Fernández et al., 2014; Reynolds et al., 2014). However, there are some inconsistencies among studies, which still require further investigation.

In all studies, there was no effect of feeding NOP on milk production. However, while not deemed significant, in all cases there was a numerical reduction in milk yield when NOP was fed, regardless of inclusion rate. While numerical changes were minimal, it is an important consideration when providing the compound to dairy cattle. Effects on body weight gain were not consistent among studies. In Study 1, cows gained more weight when fed 2,500 mg/d, however this was not found in Study 2 or by Reynolds et al. (2014). While the reason for this is unknown, each experiment was conducted under different dietary conditions and animals were at different stages of lactation which could contribute to discrepancies.

While there appears to be no negative impacts to rumen fermentation, more thorough investigation into fermentation pathways, such as propionate and hydrogen production, would be required to determine how NOP affects rumen fermentation. Feeding NOP results in a change in VFA production pattern whereby propionate proportion is increased as methane is reduced. However, this increase in propionate proportion does not account for all of the hydrogen made available through a reduction in methane, as seen in Study 1. Measuring hydrogen production, and the implications it may or may not have on the animal and digestibility is critical. Digestibility was not measured in Study 1, but Study 2 indicated there were no negative effect on digestibility, rather an increase in DM and NDF digestibility occurred. However, Reynolds et al. (2014) reported a tendency for reduced digestibility with feeding of NOP. Determining the mode of action of NOP and what happens to fermentation products such as hydrogen could provide insight as to whether or not there is an accumulation of intermediaries such as formate, which might suppress digestibility.

Overall NOP reduced methane emissions; however the extent to which they were suppressed varied among studies. For dairy studies, the greatest reduction was seen in Study 1, followed by Study 2 and Reynolds et al. (2014). While feeding method is likely the greatest factor as discussed in Chapter 2 and 3, type of diet might have affected the extent to which NOP reduced methane emissions. In Study 1, a 38% forage diet was fed and likely resulted in a lower rumen pH, although rumen pH was not measured. It has been shown that methanogens are susceptible to low rumen pH(Hook et al., 2010). Given this, it is likely in Study 1 that possible low rumen pH and effects of NOP might have synergistically acted to supress methanogens, resulting in a greater effect of NOP in comparison to Study 2 or Reynolds et al. (2014) where rumen pH was relatively high.

4.4 Future Studies

Firstly, studies should be conducted to determine whether residues of NOP or its metabolites are found in milk or meat. To build on current knowledge as to how NOP can be used to reduce methane emissions, effects of NOP should be evaluated in a long-term study. This would provide information regarding the persistency of NOP to reduce emissions, determining if there is microbial adaptation to NOP. In addition, a long-term study would provide further information regarding the effects on milk production and body weight gain. A study involving a full lactation would provide information regarding whether or not energy is retained for milk production or body weight gain, and the interaction of both. However, in the short-term determining effects on milk production should be done during early lactation when animals are in a negative energy balance. Should the reduction in methane result in more energy available to the animal, this time period is when effects are most likely to be seen. The studies conducted using lactating

cows have been relatively short (~28 d) and while suitable for detecting changes in milk production, could be too short to determine the full effect on body weight gain.

Furthermore, studies determining digestibility are necessary to obtain consistent results. A study similar to Reynolds et al. (2014) whereby all digestibility measurements are evaluated is recommended; however it would be beneficial to include the compound into the TMR. Reynolds et al. (2014) dosed NOP into the rumen which would not occur on-farm. In addition, dosing could result in a higher NOP to feed ratio present in the rumen, compared to continual consumption which could have temporary negative effects on rumen dynamics or digestibility.

4.5 Considerations

In moving forward with methane mitigation strategies, it is important to consider consumers attitude to such techniques. Consumers are increasingly concerned with the processes involved in getting food to their table, and with this come the desire for products to be more "natural". For example, in Canada from 2006 to 2012, organic food sales tripled, a growth rate not seen in other agri-food sectors (COTA, 2013). The shift to a more natural product is not only seen at a grocer level, but can be seen by large companies as well. Examples being A&W and their "better beef campaign" (A&W, 2013) where all meat is raised without the use of hormones or steroids, common growth promotants, and McDonalds wanting to source verified sustainable beef by 2016 (McDonalds, 2014). These changes often eliminate the use of production enhancing products which act to indirectly reduce methane emissions from cattle. While there is the

demand to reduce methane emissions from animal agriculture, the shift to a more natural product eliminates the ability to use feed additives that do so.

4.6 Conclusions

Overall feeding NOP reduced methane emissions in both studies without negative effects on animal production. In Study 1 there was no change in milk production however an increase in body weight gain was seen when the compound was fed. In Study 2, no changes to milk production or body weight gain were seen. Consistent effects on animal performance are yet to be established and should be investigated further. Regardless, both studies show that it is possible to reduce emissions significantly, without adverse effects on animal performance.

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